Characterization of Intracellular Calcium Stores in the Exocrine Pancreas

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een wetenschappelijke proeve
op het gebied
van de Natuurwetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
donderdag 17 november 1994
des namiddags te 3.30 uur precies
door

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geboren op 5 augustus 1965

te Oss

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Voor Christa, Jeanneke en Noëlle
Voor mijn ouders

Als reuzen aan den waterkant
staan molens in het polderland,
met armen hemelwaarts gericht
in deze wereld vol van licht

Zij staan daar als een oud geslacht,
de eeuwen door een stoere wacht,
die molens van de waterkant.
De trots en roem van Nederland.

J.H. Schuurmans Stekhoven

Contents

Chapter 1

General Introduction

The exocrine pancreas as a model system to study signal transduction in non-excitable cells

The pancreas is an organ which has both an exocrine and an endocrine function. The exocrine gland is responsible for synthesis and secretion of digestive enzymes and for fluid secretion [Bockman 1993; Kern 1993]. The exocrine cells are oriented in an acinus and secrete digestive enzymes and a NaCl-rich fluid [Kern 1993; Petersen 1993]. The enzymes and the fluid produced by these epithelial cells are delivered to the acinar lumen which is connected to the duct system. The duct cells secrete a bicarbonate-rich fluid which provides the drainage of digestive enzymes towards the duodenum [Case and Argent 1993]. The islets of Langerhans provide the endocrine functions of the pancreas and are dispersed throughout the exocrine tissue [Korc 1993]. The ß-cells in these islets produce and secrete insulin, a hormone involved in the control of glucose levels and energy metabolism.

Pancreatic acinar cells are extensively used as model to achieve a better understanding of stimulus-secretion coupling [Williams and Yule 1993]. One of the reasons why these cells are chosen as a model is that an almost homogeneous cell population can be isolated very easily in high amounts [Amsterdam and Jamieson 1974]. Therefore, these cells can be studied with biochemical techniques.

The main physiological activators of secretion are the gut hormones cholecystokinin (CCK) and secretin and the neurotransmitters acetylcholine (ACh) and vasoactive intestinal peptide (VIP) [Gardner and Jensen 1993; Williams and Yule 1993]. All known secretagogues bind to a G-protein coupled receptor and via a G-protein a mechanism is activated which generates the formation of second messengers [Gilman 1989; Sternweis and Smrcka 1992]. These receptors are plasma membrane intrinsic proteins and all possess seven hydrophobic transmembrane domains. Receptor activation promotes the exchange of GDP for GTP on the α-subunit of the heterotrimeric G-protein. Subsequently, the α-subunit dissociates from the $\beta\gamma$ complex and activates its effector system until GTP is hydrolyzed.

Two well known effector systems are involved in the regulation of secretion [Gardner and Jensen 1993; Williams and Yule 1993]. These effector systems are adenylate cyclase and phospholipase C. Activation of adenylate cyclase leads to the formation of the second messenger cAMP and results in the activation of protein kinase A. Phospolipase C mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol and inositol-(1,4,5)-trisphosphate ($Ins(1,4,5)P_3$) [Berridge and Irvine

1989; Fain 1990; Berridge 1993]. The hydrophobic messenger diacylglycerol activates protein kinase C [Nishizuka 1988; 1992] whereas the hydrophylic Ins(1,4,5)P_3 increases cytosolic Ca^{2+} levels by inducing Ca^{2+} release from an intracellular store [Streb et al. 1983; Berridge 1993], which in turn activates Ca^{2+}-calmodulin-activated kinases and phosphatases and is believed to play a direct and crucial role in the process of exocytosis.

Generally, it is accepted that CCK and ACh act through the phospholipase C route, whereas secretin and VIP activate adenylate cyclase [Williams and Yule 1993]. The stimulatory role of the phospholipase C signal transduction route in enzyme secretion is firmly established. The cAMP messenger system stimulates or potentiates enzyme secretion. VIP and secretin are capable in stimulating amylase secretion in guinea pig pancreas [Zhou et al. 1987]. In rabbit, for example, cAMP elevation alone does not influence basal enzyme secretion but is capable to potentiate CCK-induced amylase secretion [Willems et al. 1984; 1987a]. These findings also indicate that these two signalling pathways are not operating separately, but can act in a synergistic way on pancreatic acinar cell regulation.

In summary, receptor activation leads to a transduction and amplification of the first messenger. The generated second messengers stimulate enzyme secretion and activate protein kinases which in turn phosphorylate many target proteins. Although the role of these target proteins is largely unknown, they are believed to play an important role in cell regulation and stimulation of enzyme secretion [Williams and Yule 1993].

Role of the phospholipase C pathway in the stimulation of enzyme secretion

The most important secretagogues acting through this pathway are the peptide hormone CCK and the neurotransmitter ACh. The ACh receptor is of the muscarinic type and in pancreas molecular techniques revealed the presence of the M3 subtype [Peralta et al. 1987]. Recently, the primary structure of the CCK receptor has been determined by molecular cloning [Wank et al. 1992]. Both secretagogues stimulate amylase secretion in a supramaximal manner. Thus increasing concentrations of agonist stimulate secretion until the maximum rate is reached and higher concentrations partially inhibit secretion. (This is in contrast to the cAMP messenger system in guinea pig where VIP and secretin stimulate secretion until a plateau level is reached.) For both secretagogues binding studies revealed the presence of two receptors with different affinities. It is believed that occupation of the high affinity receptor has a positive correlation with secretory activity whereas occupation of the low affinity form is responsible for the down-stroke after maximal stimulation [Gardner and Jensen 1993; Williams and Yule 1993].

The transduction of the first messenger is mediated by G-proteins. Several G-

proteins have been identified in pancreatic acinar cells. Interestingly, ACh and CCK are functionally coupled to different G-proteins which indicates the presence of differential mechanisms of transduction [Schnefel et al. 1988; 1990].

Both CCK and ACh stimulate the hydrolysis of PIP_2. The production of $Ins(1,4,5)P_3$ is fast and transient. A maximum is reached within 10 sec. when high concentrations of agonist are used [Matozaki and Williams 1989; Willems et al. 1993b]. $Ins(1,4,5)P_3$ is either phosphorylated into $Ins(1,3,4,5)P_4$ and subsequently dephosphorylated into $Ins(1,3,4)P_3$ or directly dephosphorylated into $Ins(1,4)P_2$. This metabolism results in the ending of the Ca^{2+} mobilizing action since other inositol phosphates are less potent in releasing Ca^{2+} from intracellular stores [Berridge and Irvine 1989]. In fact, many inositol phosphates are known but their possible function remains to be elucidated [Berridge and Irvine 1989; Mennitti et al. 1993]. Only for $Ins(1,3,4,5)P_4$ a role in Ca^{2+} entry during sustained receptor activation has been suggested in acinar cells [Petersen 1989].

Diacylglycerol is the other messenger which is formed during PIP_2 hydrolysis [Matozaki and Williams 1989; Nishizuka 1992]. Just as for $Ins(1,4,5)P_3$, the formation of diacylglycerol is fast and transient when high agonist concentrations are used. In contrast to $Ins(1,4,5)P_3$ production, the transient rise is followed by a sustained rise in diacylglycerol levels and this rise is also observed when lower concentrations of CCK are used. Increasing evidence in various systems suggests a role of phospholipase D during the second phase of diacylglycerol production. The enzyme hydrolyses phosphatidylcholine and the produced phosphatidic acid is converted into diacylglycerol by a phosphatidic acid phosphatase [Nishizuka 1992]. It has recently been shown in pancreas that CCK receptor activation results in phospholipase D mediated phosphatidylcholine hydrolysis [Rydzewska et al. 1993].

Involvement of unknown messenger sytems in the action of CCK?

The recent developement of the CCK analogue CCK-JMV-180 has given new insights for the role of the high affinity CCK receptor [Matozaki et al. 1989; Sato et al. 1989]. This analogue is capable in activating the high affinity receptor but is an antagonist for the low affinity form in rat pancreas. CCK-JMV-180 stimulates amylase secretion and increases the cytosolic Ca^{2+} concentration but the analogue does not inhibit secretion when supraoptimal concentrations are used indicating that low affinity receptors are not stimulated by this compound. CCK-JMV-180 increases, just as CCK, cytosolic Ca^{2+} levels by mobilizing Ca^{2+} from intracellular stores. In contrast to CCK, however, CCK-JMV-180 is not capable in increasing $Ins(1,4,5)P_3$-levels above background and Ca^{2+} is

released from an Ins(1,4,5)P_3-insensitive Ca^{2+} store [Saluja et al. 1989; 1992]. Additional support for the involvement of an Ins(1,4,5)P_3-independent mechanism is obtained by using U73122, a recently developed inhibitor of phospholipase C activity [Bleasdale et al. 1990; Smith et al. 1990]. U73122 inhibits the response to CCK and carbachol but not that of CCK-JMV 180 [Yule and Williams 1992]. It is suggested that the sphingosine metabolite sphingosylphosphorylcholine mediates the action of CCK-JMV-180 [Yule et al. 1993]. However, the action of CCK-JMV-180 can not be prevented by this U73122. Other findings, however, do support the idea that CCK-JMV-180 can stimulate PIP_2 hydrolysis [Thorn and Petersen 1993].

In fibroblasts sphingosine and its related metabolite, sphingosine-1-phosphate have a mitogenic action. In addition, sphingosine-1-phosphate has a Ca^{2+} mobilizing action [Zhang et al. 1991]. Moreover, sphingosine derivates release Ca^{2+} from intracellular stores in permeabilized smooth muscle cells [Ghosh et al. 1990]. These findings indicate that additional messenger systems may be involved in signal transduction.

Elevation of cytosolic Ca^{2+} and activation of protein kinase C are both necessary to trigger digestive enzyme secretion

CCK or ACh receptor activation leads to a simultaneous Ca^{2+} mobilization and protein kinase C activation. The importance of both signalling pathways in amylase secretion has been established with compounds mimicking the action of the involved second messengers [De Pont and Fleuren-Jakobs 1984]. Cytosolic Ca^{2+} can be elevated artificially by means of Ca^{2+} ionophores, which are capable in dissipating Ca^{2+} gradients existing between the cytosol and intracellular Ca^{2+} stores and between the cytosol and the extracellular medium. Protein kinase C can constitutively be activated by the tumor promoting agent 12-O-tetradecanoylphorbol 13-acetate (TPA), which has a diacylglycerol-like structure. Incubation of acini with either the Ca^{2+} ionophore A23187 or TPA alone gives only a minor stimulation of enzyme secretion. Addition of both A23187 and TPA results in a synergistic activation of secretion and the rate of secretion is the same of that induced by the ACh receptor agonist carbachol.

Negative feedback mechanisms mediated by protein kinase C during hormonal stimulation

CCK receptor activation leads to receptor desensitization [Gardner and Jensen 1993]. Activation of the Gα subunit of the G-protein reduces CCK binding. Another important factor in the negative feedback mechanism is the role of protein kinase C. As

discussed above, protein kinase C plays a crucial role in the regulation of enzyme secretion and is capable in phosphorylating many pancreatic target proteins [Ederveen et al. 1989]. However, when protein kinase C is stimulated by phorbol ester treatment or by muscarinic receptor activation before hormonal stimulation, a desensitization of the response to CCK is observed [Gardner and Jensen 1993]. Phosphorylation of the CCK receptor is believed to be one of the mechanisms involved in desensitization. Analysis of the primary structure of the CCK receptors revealed the presence of four potential protein kinase C phosphorylation sites [Wank et al. 1992]. It is suggested for rabbit pancreas that protein kinase C stimulates the conversion of high to low affinity sites [Willems et al. 1993b]. In another study it is found that the number of high affinity sites was reduced without changing the number of low affinity sites [Honda et al. 1987]. CCK receptor phosphorylation is probably not the only mechanism responsible for this phenomenon since intracellularly located protein kinase C target proteins have been suggested [Willems 1987b; Willems et al. 1989].

The role of intracellular Ca^{2+} stores in calcium signalling

Cytosolic Ca^{2+} concentrations are now usually measured by means of Ca^{2+} fluorescent probes. One of the most extensively used probes is Fura-2. This fluorescent dye has great advantages compared to Quin-2, which was initially used to report cytosolic Ca^{2+} changes, since it has a higher selectivity for Ca^{2+} and more importantly, fluorescence can be elicited in both the Ca^{2+}-bound and -unbound form. This permits to measure Ca^{2+} concentrations independent of the concentration fura-2 [Grynkiewicz et al. 1985].

A basal cytosolic Ca^{2+} concentration of around 100 nM is found in unstimulated cells. When fura-2 loaded cells in suspension are stimulated with CCK or ACh a rapid rise of intracellular Ca^{2+} is observed within seconds and an average cytosolic concentration of 0.5 to 1 μM is reached. This rise is only transient and the intracellular Ca^{2+} concentration decreases to a lower but sustained level within a few minutes. This level is higher than the resting value and persists provided that the secretagogue receptors remains occupied with the agonist. The initial rise virtually completely depends on Ca^{2+} originating from stores of intracellular origin since the same initial rise is observed when cells are stimulated in the absence of extracellular Ca^{2+}. However, the signal returns faster and the elevated plateau level is not observed under this condition. Therefore, acinar cells depend on extracellular Ca^{2+} sources for the sustained Ca^{2+} response [Willems 1987b; Williams and Yule 1993]. The sustained Ca^{2+} influx has been demonstrated to play an important role in the maintenance of amylase secretion [Tsunoda

et al. 1990b]. The regulation of Ca^{2+} influx is, however, poorly understood [Muallem 1989; Parekh et al. 1993; Putney and Bird 1993; Randriamampita and Tsien 1993]. In Drosophila photoreceptors novel Ca^{2+} channels have been identified which mediate light-induced Ca^{2+} influx mechanisms [Hardie and Mincke 1993]. If related channels exist in vertebrates and have the same function is unknown, but Ca^{2+} influx is necessary to reload the depleted agonist-sensitive intracellular Ca^{2+} stores. It is shown that Ca^{2+} has to enter the cytosol before it is taken up into intracellular compartments [Muallem et al. 1990; Menniti et al. 1992]. Not only influx, but also efflux mechanisms are activated during stimulation. Hormonal activation of plasma membrane Ca^{2+} pumps has been demonstrated for pancreatic acinar cells [Muallem et al. 1988; Zhang et al. 1992]. Also a Na^{+}-Ca^{2+} exchange activity has been reported [Bayerdörffer et al. 1985] but is believed to be of minor importance [Muallem 1989].

Ca^{2+} signalling in individual cells

Recent developments permit to analyse Ca^{2+} behaviour in individual cells. Single cells can be analyzed by using the patch-clamp technique and by using microscopical techniques in combination with high-sensitive fluoresence detection systems. The latter technique allows to measure cytosolic Ca^{2+} in single cells or even in subcompartments when video-imaging techniques are applied. Also confocal microscopy can be used to analyse subcellular Ca^{2+} distribution patterns.

When cells were studied at the individual level it became clear that secretagogues induced oscillatory changes in the cytosolic Ca^{2+} concentration rather than producing a single Ca^{2+} transient as observed in cell suspensions. Woods and coworkers [1986] were the first who described these phenomena for hepatocytes. Ever since, Ca^{2+} oscillations have been demonstrated for many cell types, but the mechanism and the reasons why these oscillations occur are poorly understood [Berridge 1993]. Also in pancreatic acinar cells these repetitive Ca^{2+} transients have been observed by many investigators [Petersen 1993; Williams and Yule 1993].

Many models have been described to explain cytoplasmic Ca^{2+} oscillations [Meyer and Stryer 1988; Jacob 1990; Swillens and Mercan 1990; Berridge 1991; Dupont et al. 1991; Harootunian et al. 1991; Meyer and Stryer 1991; Somogyi and Stucki 1991; De Young and Keizer 1992; Allbritton and Meyer 1993; Berridge 1993; Dupont and Goldbeter 1993]. It is generally accepted that the production of $Ins(1,4,5)P_3$, and $Ins(1,4,5)P_3$-induced Ca^{2+} release is essential to initiate Ca^{2+} spiking and that intracellular Ca^{2+} stores do play a dominant role in this process. Many theoretical models have been

developed in order to describe the mechanism of Ca^{2+} oscillations. It is unknown whether the Ins(1,4,5)P_3 concentration is oscillating or is constant. In some models spiking is described by assuming only one (Ins(1,4,5)P_3-sensitive) pool, whereas in other models a two pool model is proposed. In two pool models Ca^{2+} signal propagation is explained by assuming, in analogy with muscle, a Ca^{2+}-induced Ca^{2+} release mechanism. In the latter model Ca^{2+} itself functions as a messenger to propagate the signal. However, new insights argue in favour of the importance of Ins(1,4,5)P_3 as the key signal to propagate Ca^{2+} waves, since Ins(1,4,5)P_3 diffuses 5-30 times faster than Ca^{2+} through the cytosol [Allbritton et al. 1992].

Many studies have been performed to unravel the factors determining Ca^{2+} waves and oscillations. The most important findings obtained with single cell analysis will be discussed for acinar cells below.

CCK induces repetitive Ca^{2+} spikes in a dose-dependent manner with a maximal frequency of 1.5 transients per minute [Tsunoda et al. 1990a; Willems et al. 1993a]. At low CCK concentrations the transients have the same amplitude whereas at higher concentrations repetitive cytosolic Ca^{2+} changes are preceded by a larger Ca^{2+} transient. At maximal effective concentrations only one large transient is observed. Interestingly, each cell is responding differently to CCK. It is shown that increasing concentrations CCK recruits acinar cells to respond. Therefore, when intracellular Ca^{2+} is measured in an acinar cell suspension, the signal reflects the average behaviour of all individual responding cells.

As expected, intracellular Ca^{2+} stores play a dominant role in these oscillations [Tsunoda et al. 1990a]. The extracellular Ca^{2+} source plays also a role, since during each transient a simultaneous transient Ca^{2+} influx is observed [Loessberg et al. 1991]. Efflux activity is also shown to occur synchronically with Ca^{2+} transients [Tepikin et al. 1992a; 1992b].

ACh also induces Ca^{2+} oscillations in acinar cells [Yule et al. 1991]. Interestingly, the maximal frequency of ACh-induced oscillations is higher (3 to 8 cycles per minute) and the pattern of oscillation is different compared to that induced by CCK. ACh-induced oscillations occur only in the presence of extracellular Ca^{2+}, in contrast to CCK-induced Ca^{2+} transients which continue for many minutes in the absence of extracellular Ca^{2+}. The CCK analogue CCK-JMV-180 evokes the same pattern of Ca^{2+} transients as ACh and these transients also depend extremely on extracellular Ca^{2+}. Thus, this study demonstrates again that ACh and CCK activate acini in a different manner. It is suggested that differential activation of protein kinase C explains the different spatiotemporal characteristics evoked by CCK and ACh [Yule et al. 1991; Lawrie et al. 1993]. Not only protein kinase C but also protein kinase A [Zhao et al. 1990] and cytosolic Ca^{2+} itself have been implied in the regulation of Ca^{2+} oscillations [Zhang and Muallem 1992;

Toescu et al. 1993].

As mentioned above, single cells can also be studied by means of electrophysiological techniques. In acinar cells Ca^{2+} oscillations can indirectly be detected by measuring Ca^{2+}-dependent Cl^- and non-selective cation currents [Petersen 1993]. By combining whole cell patch clamp recording with microfluorimetry it has been shown that Ca^{2+} spiking coincides with Ca^{2+}-dependent currents when higher concentrations of ACh are applied [Osipchuk et al. 1990]. Low concentrations Ach, however, only induce short lasting Cl^- currents without elevating the average cytosolic Ca^{2+} concentration. It has been suggested that these low ACh concentrations evoke local Ca^{2+} transients (see below). A great advantage of whole cell current measurements is that membrane impermeable compenents, like $Ins(1,4,5)P_3$ for example, can be introduced easily into the cell. The use of this technique has given many additional insights in the mechanism of Ca^{2+} spiking. In acinar cells Ca^{2+} oscillations can be evoked in the continuous presence of the non-metabolizable $Ins(1,4,5)P_3$ analogue inositol trisphosphorothioate $Ins(1,4,5)PS_3$ [Wakui et al. 1989]. Several reports indicate the presence of a Ca^{2+}-induced Ca^{2+} release mechanism in acinar cells [Osipchuk et al. 1990; Wakui et al. 1990]. The role of basal Ca^{2+} levels, cytosolic Ca^{2+} buffering and endoplasmic reticulum Ca^{2+} pumps during spiking has also been demonstrated [Petersen et al. 1991a; 1991b; 1993; Toescu et al. 1993].

More recent studies on the mechanism of subcellular Ca^{2+} gradients, have revealed that Ca^{2+} gradients are initiated at the luminal pole which is followed by a rise at the basolateral pole [Kasai and Augustine 1990]. This apical to basolateral Ca^{2+} wave has also been demonstrated in other acinar cell types [Elliott et al. 1992; Tan et al 1992]. It has been suggested by Kasai and Augustine that this type of Ca^{2+} gradient could trigger unidirectional Cl^- secretion and the push-pull model has been put forward to explain the observations. The Ca^{2+} rise at the luminal site activates a Cl^- efflux (push phase) and the subsequent rise at opposite pole stimulates a cotransport of Na^+ and Cl^- (pull phase). In lacrimal acinar cells the density of Ca^{2+}-dependent ion channels is about 10 times higher at the luminal membrane compared to the density at the basolateral membrane, which supports the fluid secretion model [Tan et al. 1992]. Recently, it has been demonstrated that the agonist-induced local Ca^{2+} rises can trigger exocytosis directly in these non-excitable cells [Maruyama et al. 1993]. Also in excitable cells, like melanotrophs, a rise in cytosolic Ca^{2+} is essential for the final steps in secretion [Thomas et al. 1990; 1993]. These findings argue in favour for the crucial role of cytosolic Ca^{2+} in secretory activity. It is interesting to note that chloride conductances have been demonstrated in zymogen granules of acinar cells. It is proposed that during the exocytotic event, the content of the fused granule is flushed out by electrolyte and water fluxes [De Lisle and Hopfer 1986; Thévenod et al. 1990; Piiper et al. 1991].

The rise in the luminal pole is initiated by $Ins(1,4,5)P_3$ and Ca^{2+}-induced Ca^{2+} release is believed to be the mechanism propagating the Ca^{2+} wave through the cytoplasm [Nathanson et al. 1992; Kasai et al. 1993; Thorn et al. 1993]. Detailed analysis with a high spatial resolution revealed that physiological and thus low agonist concentrations evoked local Ca^{2+} oscillations in the luminal area and this observation explains why Ca^{2+}-dependent ion currents were observed without detectable changes in the average cytosolic Ca^{2+} concentration [Osipchuk et al. 1990]. A heterogeneous distribution of Ca^{2+} channels is proposed to be the underlying mechanism explaining the Ca^{2+} waves. In this model three different Ca^{2+} stores are proposed: (i) stores with a high $Ins(1,4,5)P_3$-sensitivity located in the trigger zone in the luminal area and are surrounded by (ii) Ca^{2+}-sensitive stores and (iii) stores with a low $Ins(1,4,5)P_3$-sensitivity present in the basolateral area [Kasai et al. 1993; Thorn et al. 1993].

Electron microscopy demonstrated that CCK receptors are located on the lateral and basal plasmalemma and not on apical membranes [Rosenzweig et al. 1983]. The high diffusion rate of $Ins(1,4,5)P_3$ and the high $Ins(1,4,5)P_3$-sensitivity at the site of exocytosis are a reasonable explanation for the observed results. The low diffusion coefficient of Ca^{2+} can be an argument against Ca^{2+}-induced Ca^{2+} release as the wave propagation mechanism [Gromada et al. 1993].

The permeabilized cell as a model system to study properties of intracellular Ca^{2+} stores

As outlined above, intracellular Ca^{2+} stores play a crucial role in Ca^{2+} signalling. When intact cells are used it is difficult to study intracellular stores in more detail. In general, two techniques are used to study characteristics of intracellular stores with biochemical approaches. These techniques are (i) cell homogenization and fractionation and (ii) cell permeabilization. Fractionation is very useful in the identification of intracellular compartments. With the permeabilization technique, plasma membranes can be rendered permeable without affecting intracellular compartments. Therefore, the latter technique has great advantages when functional properties of intracellular Ca^{2+} stores are investigated. By using permeabilized acinar cells, Streb and coworkers demonstrated in 1983 for the first time that $Ins(1,4,5)P_3$ mobilizes Ca^{2+} from non-mitochondrial intracellular Ca^{2+} stores [Streb et al. 1983]. Not only $Ins(1,4,5)P_3$, but also secretagogues are capable in eliciting Ca^{2+} release. Therefore, the essential characteristics of the $Ins(1,4,5)P_3$ signalling pathway remain functionally active in this system.

Various permeabilization techniques have been described and are mainly used to study (i) the final events in exocytosis [Gomperts 1990; Knight and Scrutton 1993] and (ii) intracellular Ca^{2+} stores [Schulz 1990]. Depending on the method used pores with

different diameters can be obtained in the plasma membrane. The smallest diameters are obtained by placing cells in an electric field. Pores of a diameter of 2 nm are obtained and only ions and small molecules like nucleotides are permeant. With digitonin or saponin pore diameters of around 9 nm are obtained and enzymes up to 200 kDa can easily pass these pores. Both digitonin and saponin react with cholesterol to form pores. Since cholesterol is present in high amounts in the plasma membrane and not in intracellular membrane structures, these detergents selectively react with plasma membrane. Only when high concentrations are used, structural changes are observed in intracellular texture [Cook et al. 1983; Schulz 1990]. When low concentrations are used endoplasmic reticulum and other intracellular organelles remained intact [Wakasugi et al. 1982]. Recently, it has been shown that in digitonin permeabilized cells in suspension rearrangement of the endoplasmic reticulum structure occurs [Renard-Rooney et al. 1993]. Interestingly, however, if cells remain attached on coverslips no structural changes are observed. Finally, streptolysin-O, a bacterial pore forming toxin, creates diameters of more than 15 nm. These diameters allow the introduction of antibodies into the cell.

In studies on intracellular Ca^{2+} stores mainly digitonin and saponin are used to obtain a permeabilized system, but also electroporation is often applied. Streptolysin-O is very popular in studies where exocytotic events are investigated. The latter studies will not be discussed in detail. However, it is worth mentioning that also with this system the important role of Ca^{2+} in exocytosis is demonstrated [Knight and Koh 1983]. But other factors, like ATP and GTP are also important in the latter process since in the presence of these nucleotides amylase secretion can be stimulated by Ca^{2+} at μM concentrations [Edwardson et al. 1990].

Methods to measure Ca^{2+} fluxes

Ca^{2+} movements can be measured in two ways. The first is that the Ca^{2+} residing in compartments is measured and the second is that changes in the cytosolic Ca^{2+} concentration are used to report Ca^{2+} fluxes. For the first method usually radioactive $^{45}Ca^{2+}$ is added in tracer amounts in order to monitor the Ca^{2+} content within the vesicle [e.g. Willems et al. 1989]. This radioactive technique is often combined with a Ca^{2+} buffering system allowing a good experimental control over the free extravesicular Ca^{2+} concentration [e.g. Van Heeswijk et al. 1984]. More recently, fluorescent Ca^{2+} probes with a low affinity for Ca^{2+} have been applied to report changes in intravesicular Ca^{2+} concentrations [Hofer and Machen 1993; Renard-Rooney et al. 1993]. In addition, molecular techniques have been used to target expression of the Ca^{2+} luminescent protein aequorin in several organelles. Specific expression of recombinant aequorin is

demonstrated in mitochondria [Rizzuto et al. 1992; Rutter et al. 1993], nucleoplasm [Brini et al.] endoplasmic reticulum [Kendall et al. 1992] and (plant) cytoplasm [Knight et al. 1993]. These recent techniques make it possible to measure Ca^{2+} changes in situ. Finally, patch-clamp techniques can be used, provided that the size of the organelle is sufficiently large. This electrophysiological technique has been applied to measure $Ins(1,4,5)P_3$-induced currents in red beet vacuoles [Alexandre et al. 1990].

A second way to determine Ca^{2+} fluxes in intracellular stores is to measure the ambient free Ca^{2+} concentration with Ca^{2+} electrodes [e.g. Streb et al. 1983] or with Ca^{2+} fluorescent probes like fura-2 [e.g. Thomas 1988] or fluo-3 [e.g. Meyer and Stryer 1990]. To probe Ca^{2+} fluxes in this way cytosolic Ca^{2+} concentration has to fluctuate. Therefore, the density of the cells must be high and the medium has to be (virtually) free of Ca^{2+} chelating compounds like EGTA. In some studies radioactive Ca^{2+} efflux is measured by aspirating and changing the medium from attached permeabilized cells which Ca^{2+} stores had been loaded with $^{45}Ca^{2+}$ [e.g. Missiaen et al. 1992b].

Characterization of intracellular Ca^{2+} stores

Before the Ca^{2+} mobilizing action of $Ins(1,4,5)P_3$ was demonstrated, intracellular stores were studied in acinar cells since the importance of these stores in Ca^{2+} homeostasis and signal transduction was expected. The endoplasmic reticulum and mitochondria were proposed to be candidates to serve as a Ca^{2+} "trigger" pool. ATP-dependent Ca^{2+} accumulating properties of both mitochondria and endoplasmic reticulum were demonstrated to be present in permeabilized pancreatic acinar cells [Wakasugi et al. 1982]. The affinities of the two systems for Ca^{2+} is different. The non-mitochondrial uptake system is the system with the highest affinity for Ca^{2+}. When permeabilized cells are allowed to regulate the cytosolic free Ca^{2+} concentration, intracellular stores are able to reduce cytosolic Ca^{2+} to a value of 0.4 μM [Streb and Schulz 1983]. In absence of mitochondrial uptake activity, non-mitochondrial stores reduce the cytosolic Ca^{2+} concentration to the same value, although uptake occurs considerably slower. When only mitochondria are allowed to regulate cytosolic Ca^{2+}, the obtained steady state Ca^{2+} level is considerably higher. These results demonstrate that non-mitochondrial Ca^{2+} stores play a dominant role in cytosolic Ca^{2+} homeostasis.

Since the Ca^{2+} mobilizing action of $Ins(1,4,5)P_3$ was established, mainly non-mitochondrial Ca^{2+} stores have been studied [Streb et al. 1983]. Ca^{2+} uptake in non-mitochondrial stores is ATP-dependent, Mg^{2+}-dependent, pH-sensitive and is modulated by submicromolar concentrations Ca^{2+} [Bayerdörffer et al. 1984; Richardson and Dormer 1984; Hurley 1988]. All Ca^{2+} uptake in non-mitochondrial Ca^{2+} stores in pancreatic

acinar cells is thought to be provided by Ca^{2+}, Mg^{2+}-ATPases. Recently, it has been demonstrated that the endoplasmic reticulum Ca^{2+} pump is of the SERCA-2b type [Dormer et al. 1993]. The apparent affinity for Ca^{2+} under optimal conditions has a value between 0.15 and 0.5 μM. These values correlate well with the K_m of 0.27 μM Ca^{2+} reported for SERCA2b pumps transiently expressed in COS cells [Lytton et al. 1992]. Not only Ca^{2+} pumps, but also proton pumps have been shown to be involved in the uptake of Ca^{2+} in Ins(1,4,5)P_3-sensitive microsomal vesicle fractions of pancreatic acinar cells [Thévenod et al. 1987; 1989].

Cell fractionation demonstrated that Ins(1,4,5)P_3-sensitivity copurifies with markers of the endoplasmic reticulum [Streb et al. 1984]. Therefore, microsomal fractions are also often used to study Ins(1,4,5)P_3-sensitive Ca^{2+} stores [Dawson and Comerford 1989; Gill et al. 1989; Schulz et al. 1989]. Further fractionation of these stores revealed a heterogeneous distribution of Ins(1,4,5)P_3-sensitive Ca^{2+} stores [Dehlinger-Kremer et al. 1991]. One of the major disadvantages of microsomes is that the size of the Ins(1,4,5)P_3-sensitive compartments is markedly reduced compared to the large Ins(1,4,5)P_3-sensitivity in permeabilized cells [Dawson and Comerford 1989]. Additional release mechanisms have been demonstrated with microsomal preparations. It has been shown that GTP induces Ca^{2+} release in a polyethylene glycol dependent manner. GTP hydrolysis is required to observe this release since GTPγS is a competitive inhibitor. At low ambient free Ca^{2+} concentrations of 0.1 μM GTP does not release Ca^{2+} but increases enourmously Ins(1,4,5)P_3-sensitivity in liver microsomal preparations [Dawson and Comerford 1989]. Also in several permeabilized cell systems GTP increases the size of the Ins(1,4,5)P_3-sensitive pool whereas in other cell types the effects of GTP and Ins(1,4,5)P_3 are additive. GTP can induce vesicle fusion and it has been proposed that this results in a coupling between vesicles only containing pumps and vesicles only containing Ins(1,4,5)P_3-receptors [Dawson and Comerford 1989; Hampe et al. 1990]. In other systems, vesicle fusion is not demonstrated but a GTP-induced coupling between compartments was shown to occur [Gill et al. 1989]. Small GTP-hydrolyzing proteins which are involved in membrane traffic are likely candidates to mediate the action [Bourne 1989; Bourne et al. 1990]. Recently, an ADP-ribosylation factor was identified as a possible candidate to be involved in membrane traffic in acinar cells [Zeuzem et al. 1992]. In conclusion, a GTP hydrolysing mechanism is believed to mediate communication between intracellular Ca^{2+} stores and/or to modulate the size of the Ins(1,4,5)P_3-sensitive pools.

Molecular properties of the Ins(1,4,5)P_3-receptor

The Ins(1,4,5)P_3-receptor complex is comprised of four homologous subunits of 260 kDa [Snyder and Supattapone 1989; Chadwick et al. 1990; Ferris and Snyder 1992]. The primary structure of the cerebellar Ins(1,4,5)P_3 receptor was identified in 1989. The receptor shares homology with the skeletal muscle ryanodine receptor and several subtypes have been reported [Furuichi et al. 1989; Mignery et al. 1989; Ferris and Snyder 1992]. Initially, only protein kinase A phosphorylation sites were identified on the Ins(1,4,5)P_3-receptor [Ferris et al. 1991a]. Further studies demonstrated that also protein kinase C and Ca^{2+}/calmodulin dependent kinases are able to phosphorylate this receptor [Ferris et al. 1991b].

Modulation of Ins(1,4,5)P_3-induced Ca^{2+} release by protein kinases

In brain, phosphorylation of the Ins(1,4,5)P_3 receptor by protein kinase A results in a reduced potency of Ins(1,4,5)P_3 to mobilize Ca^{2+}. In liver, however, protein kinase A has a potentiating action on the Ins(1,4,5)P_3-induced Ca^{2+} mobilization and on the binding characteristics of the Ins(1,4,5)P_3-receptor [Hajnóckzy et al 1993; Joseph and Ryan 1993]. Also protein kinase C activation modulates Ins(1,4,5)P_3-induced Ca^{2+} release. Pretreatment of intact acinar cells with phorbol ester or hormone results in a reduced Ins(1,4,5)P_3-sensitivity in the permeabilized system. It has been suggested that phosphorylation of the Ins(1,4,5)P_3-receptor mediates these effects and that also this mechanism is one of the negative feed-back loops of protein kinase C on acinar cells (see above). Recently, Muallem and coworkers have demonstrated that calmodulin-dependent kinases and -phosphatases can control the phosphorylation state and thereby properties of Ins(1,4,5)P_3-induced Ca^{2+} release [Zhao and Muallem 1990; Zhang et al. 1993].

Quantal Ca^{2+} release and other properties of the Ins(1,4,5)P_3-induced Ca^{2+} release mechanism

An intriguing property of Ins(1,4,5)P_3-induced Ca^{2+} release is its quantal nature. Muallem and co-workers [1989] demonstrated for the first time this feature by using permeabilized pancreatic acinar cells. It was observed that a submaximal dose of Ins(1,4,5)P_3 released only a fraction of the Ca^{2+} stored within Ins(1,4,5)P_3-sensitive compartments. It was concluded that this "quantal" release of Ca^{2+} from intracellular stores was the mechanism by which hormones evoke Ca^{2+} release in intact cells. This

"quantal" behaviour to agonist stimulation was shown to occur in single HeLa and chromaffin cells and again it was concluded that the "quantal" release of Ca^{2+} from the intracellular stores was the underlying mechanism [Bootman et al. 1992; Cheek et al. 1993].

Some theoretical models have been developed [Tregear et al. 1991; Swillens 1992; Kindman and Meyer 1993] and many studies have been performed to elucidate the mechanism by which the Ins(1,4,5)P_3-receptor opens and how release occurs. In contrast to neurotransmitter-operated ion channels (e.g. nicotinic acetylcholine receptor [Ferris et al. 1992]), the Ins(1,4,5)P_3 receptor is not desensitized since subsequent addition of Ins(1,4,5)P_3 results in further Ca^{2+} release [Taylor and Potter 1990; Oldershaw et al. 1992]. Moreover, if a maximal effective concentration of Ins(1,4,5)P_3 is added after a submaximal dose, complete release of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool is observed. In permeabilized rat hepatocytes this behaviour does not depend on the presence of pump activity since the same properties are observed in the absence of pump activity. Quantal release occurs, as suggested originally by Muallem and coworkers, in an "all-or-nothing" fashion, i.e. during submaximal stimulation the most sensitive stores are depleted completely whereas the less sensitive stores remain unaffected [Oldershaw et al. 1991]. Only an increase in the Ins(1,4,5)P_3 concentration can completely mobilize Ca^{2+} from the less sensitive stores. In other words, the Ins(1,4,5)P_3-sensitive Ca^{2+} compartments are a heterogeneous population of Ca^{2+} stores. A heterogeneity among Ins(1,4,5)P_3-receptors may explain these observations since the "quantal" kinetics of Ins(1,4,5)P_3-induced fluxes are preserved in purified Ins(1,4,5)P_3-receptors reconstituted in lipid vesicles [Ferris et al. 1992].

In other permeabilized cell systems, however, it is demonstrated that in the absence of pump activity, Ca^{2+} release is a steady-state phenomenon [Missiaen et al. 1992b; Loomis-Husselbee and Dawson 1993]. The latter implies that in the presence of pump activity release is occurring in a "quantal" fashion and that after this release a new equilibrium is created between pump activity and the increased Ca^{2+} permeability induced by Ins(1,4,5)P_3. These results suggest, as a consequence, incomplete emptying of Ins(1,4,5)P_3-sensitive stores during submaximal stimulation.

The mechanism explaining Ins(1,4,5)P_3-induced Ca^{2+} release and especially the mechanism controlling the opening activity of the Ins(1,4,5)P_3-operated Ca^{2+} channel is, however, still poorly understood. Under experimental conditions where both the intravesicular side and the cytosolic side are well under control, the opening kinetics of the Ins(1,4,5)P_3-receptor do not inactivate [Ehrlich and Watras 1988; Alexandre et al. 1990]. Rapid kinetic studies in permeabilized basophilic leukemia cells revealed that the initial response was highly cooperative [Meyer et al. 1988, 1990]. It was proposed that all four subunits of the Ins(1,4,5)P_3-operated Ca^{2+} conducting channel have to bind

$Ins(1,4,5)P_3$ before opening can occur. Also in this system quantal amounts of Ca^{2+} are released by submaximal concentrations of $Ins(1,4,5)P_3$. Subsequent additions of higher but still submaximal amounts of $Ins(1,4,5)P_3$ result in a further release. Since inactivation or adaptation of the release process does not occur, the phenomenon has been termed "increment detection". The high cooperative nature of channel opening is supposed to be an essential requirement to explain quantal release [Meyer and Stryer 1990]. In permeabilized hepatocytes, however, a fast kinetic study did not confirm the cooperative nature of initial Ca^{2+} efflux [Champeil et al. 1989]. Release is not mono-phasic but is composed of a fast and a slow component. In contrast to the findings reported for leukemia cells, the kinetics have a moderate temperature dependence. In order to explain the biphasic kinetics and quantal release in hepatocytes it was suggested that the $Ins(1,4,5)P_3$ receptor can occur in two conductance states.

Role of intravesicular Ca^{2+} in the regulation of $Ins(1,4,5)P_3$-induced Ca^{2+} release

In 1990 Irvine proposed a hypothetical role for the intravesicular Ca^{2+} in the control of $Ins(1,4,5)P_3$-induced Ca^{2+} release. It was proposed that increasing concentrations luminal Ca^{2+} sensitize the $Ins(1,4,5)P_3$-receptor for $Ins(1,4,5)P_3$ [Irvine 1990]. This model predicts that loaded stores respond to a sub-optimal dose of $Ins(1,4,5)P_3$ and that release from the $Ins(1,4,5)P_3$-sensitive store stops if the Ca^{2+} concentration drops below a certain level. Therefore, depleted stores should be unresponsive to the same sub-optimal concentration of $Ins(1,4,5)P_3$.

In several reports on different cell types the Ca^{2+} content was manipulated by various means but a uniform answer about the role of the intravesicular Ca^{2+} concentration has not been obtained yet. For some tissues luminal Ca^{2+} has a role whereas in other systems luminal Ca^{2+} has no or a controversial role. For smooth muscle a clear role for luminal Ca^{2+} has been established [Missiaen et al. 1992b; 1992c; 1994; Parys et al. 1993] whereas for hepatocytes no uniform answer is obtained [Missiaen et al. 1991; Combettes et al. 1992; Missiaen et al. 1992a; Nunn and Taylor 1992; Combettes et al. 1993; Marshall and Taylor 1993; Oldershaw and Taylor 1993]. For exocrine nasal gland cells, however, no role for luminal Ca^{2+} is found [Shuttleworth 1992]. The findings will be discussed in more detail below.

Missiaen and coworkers have reported several roles for luminal Ca^{2+} in the process of $Ins(1,4,5)P_3$-induced Ca^{2+} release. They have demonstrated for permeabilized A7r5 smooth muscle cells that the pattern of $Ins(1,4,5)P_3$-induced Ca^{2+} release is controlled by luminal Ca^{2+} [Missiaen et al. 1992b]. During these experiments, release was studied in the absence of Ca^{2+} pump activity and in the absence of cytosolic Ca^{2+}.

The pattern of release consists of a fast component followed by a slow component which slows down when stores become more depleted. Release stops when the Ca^{2+} content within the store is decreased to a certain critical level indicating that luminal Ca^{2+} controls the opening activity of the Ins(1,4,5)P_3-operated Ca^{2+} channel. In their next study the role of luminal Ca^{2+} was studied in the presence of cytosolic Ca^{2+} [Missiaen et al. 1992c]. It was shown that the presence of cytosolic Ca^{2+} potentiates Ins(1,4,5)P_3-induced Ca^{2+} release. Interestingly, the effect of cytosolic Ca^{2+} becomes more important when Ca^{2+} stores are relatively more depleted. In recent studies, the Ca^{2+} content within the stores was manipulated by using different loading protocols [Parys et al. 1993; Missiaen et al. 1994]. The same effects of luminal and cytosolic Ca^{2+} were found and in addition it was reported that increasing vesicular Ca^{2+} concentrations increase Ins(1,4,5)P_3-binding. The expression of several subtypes of Ins(1,4,5)P_3-receptors in this cell type may account for the observed release characteristics in smooth muscle.

In hepatocytes the role of luminal Ca^{2+} is still controversial. Combettes and coworkers found no effect, whereas Taylor and coworkers found a role for luminal Ca^{2+} in the control of Ins(1,4,5)P_3-induced Ca^{2+} release [Combettes et al. 1992; Nunn and Taylor 1992; Combettes et al. 1993; Oldershaw and Taylor 1993]. Taylor and coworkers used different concentrations ionomycin to manipulate the store size and found a minor but significant increase in the EC_{50} for Ins(1,4,5)P_3-induced Ca^{2+} release [Nunn and Taylor 1992] and for Ins(1,4,5)P_3-binding characteristics [Oldershaw and Taylor 1993]. In a more recent study, by using the SERCA Ca^{2+}-ATPase inhibitor thapsigargin [Lytton et al. 1991] as a tool to deplete Ca^{2+} stores, no effect of store depletion on the relative efficiency of Ins(1,4,5)P_3 to release Ca^{2+} was found [Marshall and Taylor 1993].

Role of cytosolic Ca^{2+} in the regulation of Ins(1,4,5)P_3-induced Ca^{2+} release

Cytosolic Ca^{2+} is known to modulate Ins(1,4,5)P_3-induced Ca^{2+} release. In smooth muscle the effects of cytosolic Ca^{2+} depend on the filling state of the Ca^{2+} stores (see above). In pancreatic acinar cells ambient free Ca^{2+} concentrations above 1 μM inhibit Ins(1,4,5)P_3-induced Ca^{2+} release [Willems et al. 1990]. More recent studies have shown that cytosolic Ca^{2+} can exert both stimulatory and inhibitory effects on Ins(1,4,5)P_3 binding and Ins(1,4,5)P_3-induced Ca^{2+} release. Mauger and co-workers characterized in detail Ins(1,4,5)P_3-binding sites in hepatocytes [Mauger et al. 1989; Pietri et al. 1990; Pietri-Rouxel et al. 1992]. These studies revealed the presence of two interconvertable binding sites, a high- and and a low-affinity binding site. Hormonal pretreatment of intact cells induces the conversion of low-affinity binding sites into high-affinity sites. The hormonal effect is likely to be mediated by cytosolic Ca^{2+} since submicromolar Ca^{2+}

concentrations induce the same conversion in permeabilized hepatocytes. Interestingly, this conversion results in a reduced ability of $Ins(1,4,5)P_3$ to release Ca^{2+}. Therefore, the low-affinity conformation of the $Ins(1,4,5)P_3$ receptor is associated with Ca^{2+} release whereas the high-affinity form occurs in a closed state.

Beside the inhibitory action, cytosolic Ca^{2+} can also potentiate $Ins(1,4,5)P_3$-induced Ca^{2+} release. The open probability for $Ins(1,4,5)P_3$-receptors, reconstituted in planar lipid bilayers, is modulated in a bell-shaped fashion by cytosolic Ca^{2+} [Bezprozvanny et al. 1991]. Maximum opening occurs at 0.2 μM and a sharp decrease is observed at either side of the maximum. By using a rapid superfusion system Finch et al. [1991] have investigated the role of cytosolic Ca^{2+} on $Ins(1,4,5)P_3$-induced Ca^{2+} release in synaptosome-derived microsomal vesicles. This system allows to study release with a high temporal resolution. It was shown that cytosolic Ca^{2+} potentiates and stimulates release with an EC_{50} of 0.7 μM. Beside this co-agonistic action, cytosolic Ca^{2+} reduces the maximal releasable amount of Ca^{2+} with an IC_{50} of 0.6 μM. Interestingly, the inhibitory effect developes more slowly than the potentiating effect. The inhibitory effect is reversible and is independent of $Ins(1,4,5)P_3$ since a prepulse of Ca^{2+} diminishes the $Ins(1,4,5)P_3$ response. The inhibitory effect of Ca^{2+} may therefore be explained by the two-state model for the $Ins(1,4,5)P_3$ receptor described above. Marshall and Taylor [1993] also demonstrated biphasic effects of cytosolic Ca^{2+} in permeabilized hepatocytes. To explain the stimulatory effect of cytosolic Ca^{2+} the two affinity state model of Mauger and coworkers was extended by assuming an open state of the high affinity receptor.

For other peripheral tissues, including the pancreatic acinar cell line A42J, it was shown by Muallem and coworkers that cytosolic Ca^{2+} modulates $Ins(1,4,5)P_3$-induced Ca^{2+} release in a biphasic manner by activating Ca^{2+}/calmodulin dependent kinases. Lower concentrations of Ca^{2+} stimulate $Ins(1,4,5)P_3$-induced Ca^{2+} release with an apparent affinity of 15 nM [Zhang et al. 1993]. Higher Ca^{2+} concentrations inhibit release with an apparent affinity of 0.24 μM [Zhao and Muallem 1990]. The stimulatory and the inhibitory effect appear to be mediated by a Ca^{2+}-dependent kinase and phosphatase respectively. It was therefore suggested that cytosolic Ca^{2+} controls $Ins(1,4,5)P_3$-induced Ca^{2+} release by controlling the phosphorylation state of the receptor.

In summary, the molecular mechanisms underlying the opening activity of $Ins(1,4,5)P_3$-operated Ca^{2+} conducting channels and $Ins(1,4,5)P_3$-induced Ca^{2+} release are very complex. Receptor and release properties can be modulated by cytosolic Ca^{2+}, intravesicular Ca^{2+} and by protein kinases and phosphatases. These modulatory factors are believed to be involved in the regulation of cytosolic Ca^{2+} transients in intact cells, since they provide a system which can rapidly switch on and off. Especially the off switch is very poorly understood. A better understanding of this part of the regulation is very important since refilling of Ca^{2+} stores have to proceed a new spike.

Are a Ca^{2+}-induced Ca^{2+} release mechanism and ryanodine receptors present in acinar cells?

Ca^{2+}-induced Ca^{2+} release is believed to be the underlying mechanism by which Ca^{2+} is mobilized in skeletal muscle [Fabiato 1983; McPherson and Campbell 1993]. The Ca^{2+} channel mediating this release is the ryanodine receptor which is homologous with the Ins(1,4,5)P_3 receptor. The action of Ca^{2+} is potentiated by caffeine and caffeine-induced Ca^{2+} release is often seen as an indication for the existence of such a mechanism in non-muscle cells [Cheek et al. 1993]. Recent developments suggest that a metabolite of NAD^+, cyclic ADP-ribose, is an endogeneous modulator of Ca^{2+}-induced Ca^{2+} release in many cells [Rusinko and Lee 1989; Galione et al. 1991; Galione 1992; Lee 1993]. Studies on individual acinar cells have given supportive evidence for existence of such a mechanism and it is suggested that this mechanism plays an important role in the propagation of the Ca^{2+} wave [Nathanson et al. 1992; Kasai et al. 1993; Petersen 1993]. Until present there is no clear demonstration of Ca^{2+}-induced Ca^{2+} release in permeabilized pancreatic acinar cells (and many other non-muscle cells). The use of caffeine has its restrictions because it can inhibit Ins(1,4,5)P_3-induced Ca^{2+} release [Brown et al. 1992] and can block Ins(1,4,5)P_3 production [Toescu et al. 1992]. Moreover, the positive effects of cytosolic Ca^{2+} on Ins(1,4,5)P_3-induced Ca^{2+} release may also be interpreted as Ca^{2+}-induced Ca^{2+} release when Ins(1,4,5)P_3-levels are constant. In conclusion, further studies in non-muscle cells are needed to elucidate the existence and mechanism of Ca^{2+}-induced Ca^{2+} release.

Localization and composition of Ins(1,4,5)P_3-sensitive and other intracellular Ca^{2+} stores

In a morphological study, Volpe and coworkers [1988] presented evidence for the presence of calsequestrin-like proteins in the intravesicular lumen of Ca^{2+} accumulating compartments in non-muscle tissue. Calsequestrin acts in striated muscle as a Ca^{2+} buffer since it can bind up to 50 molecules of Ca^{2+} with a moderate affinity of 1 mM [MacLennan et al. 1983]. Calsequestrin is, just as the ryanodine receptor, located in the terminal cisternae of the sarcoplasmic reticulum [Franzini-Armstrong et al. 1987] whereas Ca^{2+}-ATPases are mainly located in the longitudinal portion [Jorgensen et al. 1982]. This distribution indicates that the sites of accumulation are separated from the sequestration and release sites. The calsequestrin-like protein was recently identified as calreticulin and was shown to possess the same characteristics as calsequestrin [Treves et al. 1990].

It was originally suggested that the calsequestrin-like protein is located in an organelle distinct from endoplasmic reticulum or other known organelles [Volpe et al. 1988]. It was hypothesized that this "calciosome" was equipped with Ca^{2+} pumps, Ins(1,4,5)P_3-receptors and a Ca^{2+} sequestering system. Further studies in cerebellum and smooth muscle indicate that the Ins(1,4,5)P_3-receptor is localized in a subcompartment of the endoplasmic reticulum [Satoh et al. 1990; Villa et al. 1993]. In non-muscle cells, like hepatocytes and pancreas, the presence of Ins(1,4,5)P_3 receptors has, however, never been demonstrated by means of morphological techniques [Hashimoto et al. 1988]. The mechanism of subcellular Ca^{2+} gradients in exocrine pancreas suggests the presence of Ins(1,4,5)P_3-sensitive stores at both the basal and the luminal pole of the cell and the stores at the apical pole are localized close to the plasma membrane [Kasai et al. 1993; Thorn et al. 1993]. Recent developements allow to measure intravesicular Ca^{2+} movements in permeabilized systems [Renard-Rooney et al. 1993; Short et al. 1993]. In hepatocytes it was found that Ins(1,4,5)P_3 induced a release of Ca^{2+} from organelles throughout the cytoplasm but not from the nuclear portion of the cell [Renard-Rooney et al. 1993]. In smooth muscle, however, Ins(1,4,5)P_3-sensitive stores are localized around the nucleus and close to the cell periphery [Short et al. 1993].

Many fractionation studies have been performed in order to purify the Ins(1,4,5)P_3-sensitive store. During initial studies, Ins(1,4,5)P_3-sensitivity was shown to correlate with markers of the endoplasmic reticulum [Streb et al. 1984]. Further studies with pancreatic and other peripherial tissues suggest a heterogeneous distribution of Ins(1,4,5)P_3-binding sites in pancreas since binding is not only correlated with markers for the rough endoplasmic reticulum but also with plasma membrane markers [Rossier and Putney 1991, Sharp et al. 1992]. Recently, Ins(1,4,5)P_3-receptors were found to be closely associated with ankyrin, indicating that the cytoskeleton may be involved in the structural localization of Ca^{2+} stores [Bourguignon et al. 1993; Joseph and Samanta 1993]. Ins(1,4,5)P_3-sensitive stores are apparently equipped with a sequestration system since Ins(1,4,5)P_3-binding copurified with calreticulin [Van Delden et al. 1992; Enyedi et al. 1993].

The interactions between Ins(1,4,5)P_3-receptors, Ca^{2+} pumps and Ca^{2+} sequestration systems are suggested to exist but the exact interplay between the components of the intracellular Ca^{2+} storage system is largely unknown. In parotid acinar cells it is suggested, that in analogy with muscle, the pumps sites are segregated from the release sites [Menniti et al. 1991]. The role of calreticulin and other luminal Ca^{2+} binding proteins in Ca^{2+} homeostasis is, however, poorly understood [Michalak et al. 1992].

The aim of the study was to characterize intracellular Ca^{2+} stores and to elucidate mechanisms of Ca^{2+} release from these stores in exocrine pancreas. For this purpose, the permeabilized cell system was applied and Ca^{2+} movements were followed with radioactive tracer under strong control of the ambient free Ca^{2+} concentration.

The properties of $Ins(1,4,5)P_3$-sensitive and -insensitive Ca^{2+} accumulating compartments were investigated in more detail in *chapter 2*. It was found that GTP released Ca^{2+} from an $Ins(1,4,5)P_3$-insensitive compartment. Although GTP released Ca^{2+} independently from $Ins(1,4,5)P_3$, indications for a coupling between these stores in intact acinar cells were obtained.

Chapter 3 describes the effects of ruthenium red on the accumulation properties of $Ins(1,4,5)P_3$-sensitive and -insensitive stores. It will be demonstrated that ruthenium red, which is described as an inhibitor of Ca^{2+}-ATPases, selectively hinders the accumulation of Ca^{2+} in $Ins(1,4,5)P_3$-sensitive stores. Evidence will be provided that the action of ruthenium red can not be explained by inhibition of Ca^{2+} pumps alone.

Chapter 4 describes the effects of the SERCA Ca^{2+}-ATPase inhibitor thapsigargin on Ca^{2+},Mg^{2+}-dependent and Mg^{2+}-dependent enzyme activities in rat liver microsomes. It will be shown that thapsigargin selectively and non-competitively inhibits Ca^{2+},Mg^{2+}-ATPase activity. It will further be shown that thapsigargin is a very useful tool in the study of other ATP-hydrolyzing activities.

Chapter 5 describes the action of the phospholipase C inhibitor U73122 on intact and permeabilized acinar cells. It will be demonstrated that U73122 can deplete, just like ruthenium red, the $Ins(1,4,5)P_3$-sensitive Ca^{2+} pool in permeabilized cells. Also in intact cells U73122 selectively depletes the agonist sensitive Ca^{2+} store and this depletion results in activation of Ca^{2+} influx mechanisms.

A study on the underlying mechanisms of "quantal" Ca^{2+} release will be presented in *chapter 6*. It will be demonstrated that store heterogeneity and compensatory Ca^{2+} pumping determine the "quantal" release properties evoked by submaximal effective concentrations $Ins(1,4,5)P_3$.

Evidence for a heterogeneous distribution of Ca^{2+} pump, Ca^{2+} sequestration and release sites, will be presented in *chapter 7*. It will be demonstrated that the major site of Ca^{2+} sequestration is residing in $Ins(1,4,5)P_3$-sensitive compartments and that major pump activity is found within $Ins(1,4,5)P_3$-insensitive compartments. This study together with the study presented in *chapter 2*, indicate that in intact cells $Ins(1,4,5)P_3$-sensitive and -insensitive compartments form a continuous Ca^{2+} storage organelle.

Chapter 2

GTP-Sensitivity of the energy-dependent Ca^{2+} storage pool in permeabilized acinar cells

In: Cell Calcium, 12, 587 - 598 (1991)

CELL CALCIUM

Cell Calcium (1991) 12 587–598

GTP-Sensitivity of the energy-dependent Ca^{2+} storage pool in permeabilized pancreatic acinar cells

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Abstract — Isolated rabbit pancreatic acinar cells, permeabilized by saponin treatment and incubated in the presence of 0.1 μM free Ca^{2+}, accumulated 3.3 nmol of Ca^{2+}/mg of acinar protein in an energy-dependent pool. Part of this energy-dependent pool could be released by GTP in a polyethylene glycol-dependent manner. The kinetics of GTP-induced release of Ca^{2+} showed a biphasic pattern with an initial rapid phase followed by a sustained slower phase. In contrast, IP_3-induced release of Ca^{2+} was completed within 30 s following addition of IP_3. No reuptake of Ca^{2+} was observed following GTP- or IP_3-induced release of Ca^{2+}. The GTP effect was independent of IP_3 and not inhibited by Ca^{2+}, indicating that the IP_3-operated Ca^{2+} channel is not involved in GTP-induced release of Ca^{2+}. The size of the IP_3-releasable pool was not affected by GTP, indicating that GTP, when added to permeabilized acinar cells, does not promote the coupling between IP_3-insensitive and IP_3-sensitive Ca^{2+} accumulating organelles. Thus, in permeabilized acinar cells, GTP and IP_3 act on different Ca^{2+} sequestering pools. Interestingly, however, comparison of the size of the GTP-releasable pool with that of the IP_3-releasable pool for the cell preparations used in the present study, revealed an inversed relationship, indicating that at the time of permeabilization the GTP-releasable pool can be coupled to a greater or lesser extent to the IP_3-releasable pool. This suggests that, in the intact cell, a GTP-dependent mechanism may exist that controls the size of the IP_3-releasable pool by coupling IP_3-insensitive to IP_3-sensitive organelles. Moreover, this suggests that the extent of coupling is preserved during permeabilization.

Pancreatic secretagogues such as acetylcholine and cholecystokinin stimulate digestive enzyme secretion by activating a phosphatidylinositol 4,5-bisphosphate-specific phosphodiesterase catalyzing the formation of the intracellular messengers 1,2-diacylglycerol [1, 2] and inositol 1,4,5-trisphosphate [2]. A second messenger role for inositol 1,4,5-trisphosphate (IP_3) was proposed for the first time by Streb et al. [3]. Using permeabilized pancreatic acinar cells, they showed that IP_3 rapidly released Ca^{2+} from an energy-dependent vesicular store. In subsequent subfractionation studies, this IP_3-releasable Ca^{2+}

Abbreviations used IP_3, inositol 1,4,5 trisphosphate PEG, polyethylene glycol HEEDTA, N-(2-hydroxyethyl) ethylenediamine-N,N′,N′ triacetic acid and HEPES, 4-(2-hydroxyethyl) 1 piperazine ethanesulfonic acid

store was found to be recovered in the microsomal fraction, indicating its non-mitochondrial and non-nuclear origin [4, 5] Moreover, subcellular fractionation studies revealed that only part of the microsomal fraction is sensitive to IP_3 [6]

In some cell types, including the pancreatic acinar cell, immunocytochemical studies have been performed to characterize the energy dependent and IP_3 releasable Ca^{2+} store in more detail Localization studies using anti calsequestrin and anti-muscle sarcoplasmic reticulum Ca^{2+} ATPase antibodies suggested the existence of immunoreactive organelles distinct from the endoplasmic reticulum, referred to as calciosomes [7, 8] Subcellular fractionation revealed the co-purification of the calsequestrin like protein with markers of the IP_3-sensitive Ca^{2+} store, indicating that the calciosome might be the site of IP_3 action [7] Recently, however, using antibodies raised against the putative IP_3 receptor protein, Satoh et al [9] demonstrated that in the cerebellar Purkinje cell a large, smooth-surfaced subcompartment of the endoplasmic reticulum contained high concentrations of IP_3 receptors

In many studies using permeabilized cells, including the pancreatic acinar cell [10–12], it has been shown that the non-mitochondrial ATP-dependent Ca^{2+} store is only in part sensitive to IP_3 (for a review, *see* [13]) Recent work on intact chromaffin cells by Burgoyne et al [14], suggested that part of the IP_3 insensitive Ca^{2+} pool is releasable by caffeine and could, therefore, be the site of the Ca^{2+} induced Ca^{2+} release (for a review, *see* [13]) The latter process is thought to mediate the Ca^{2+} waves recently visualized by means of Ca^{2+} imaging techniques [15]

In some cell types, the IP_3-insensitive pool has been shown to be releasable by low concentrations of GTP (for a review, *see* [16, 17]) The mechanism of action of GTP is still unclear but it has been speculated that the GTP sensitive pool is the same as the caffeine sensitive pool [18] Interestingly, a voltage-gated Ca^{2+} channel sensitive to caffeine has recently been shown to be present in a microsomal preparation of rat pancreatic acinar cells, which was enriched in endoplasmic reticular membranes [18] For some preparations it has been demonstrated that GTP can enlarge the IP_3-releasable store, indicating the existence of mechanisms activating communication between Ca^{2+} pools insensitive and sensitive to IP_3 [19, 20]

In the present study we investigated the possibility of such a GTP-dependent regulatory mechanism to be present in the pancreatic acinar cell It is shown that permeabilized acinar cells contain a GTP-sensitive pool which can be released by GTP in the absence of IP_3 The data presented suggest that, in the intact acinar cell, a GTP-dependent mechanism exists that regulates the extent of coupling between this GTP-sensitive pool and the IP_3-releasable pool

Materials and Methods

Pancreatic acinar cells

Rabbit pancreatic acinar cells were prepared essentially as described by Amsterdam & Jamieson [21] The method is based on two successive incubations with collagenase and hyaluronidase in a Krebs Ringer bicarbonate medium (pH 7 4) containing 119 mM NaCl, 3 5 mM KCl, 1 2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0 1 mM $CaCl_2$, 1 2 mM $MgCl_2$, 5 8 mM glucose, 0 2 mg soya-bean trypsin inhibitor/ml and an amino acid mixture (Eagle [22]), with, in between, a bivalent cation chelating step After digestion, the tissue was dispersed by pipetting, filtered through nylon gauze, and purified by centrifugation through a 4% (w/v) albumin layer The intactness of the isolated acinar cells was demonstrated by Trypan Blue exclusion

Isolated pancreatic acinar cells, resuspended in the above Krebs-Ringer bicarbonate medium containing 1 0 mM $CaCl_2$ and 1% (w/v) bovine serum albumin, were incubated for 30 min at 37°C

Permeabilization of acinar cells

Isolated pancreatic acinar cells were washed twice, resuspended in a high K^+ medium (1 mg of protein/ml) containing 135 mM KCl, 1 0 mM $MgCl_2$, 1 2 mM KH_2PO_4, 0 1 mM phenylmethyl- sulfonyl fluoride, 0 2 mg of soya-bean trypsin inhibitor per ml and 10 mM HEPES (pH 7 4) and permeabilized with saponin (30 µg/ml for 10 min at 25°C After

saponin treatment, less than 25% of the cells excluded Trypan Blue

Ca^{2+} uptake and release experiments

Permeabilized acinar cells were washed twice and resuspended in a 'Ca^{2+}-uptake medium' (4 mg of protein/ml) containing 120 mM KCl, 1 0 mM $MgCl_2$, 1.2 mM KH_2PO_4, 5 mM pyruvate, 5 mM succinate, 0.5 mM EGTA, 0.5 mM nitrilotriacetic acid, 0.5 mM HEEDTA, 0.2 mg of soya-bean trypsin inhibitor per ml and 20 mM HEPES and adjusted to pH 7.1 with KOH. The mitochondrial Ca^{2+}-uptake inhibitor Ruthenium Red (20 μM) was added, and the permeabilized cells were kept on ice for 1h. Ca^{2+} uptake was started by adding 10 μl of permeabilized cells to 90 μl of Ca^{2+}-uptake medium, which contained in addition. 10 mM creatine phosphate, 10 units of creatine kinase per ml, 20 μM-Ruthenium Red, 0 or 1 mM NaATP, 0 or 3% polyethylene glycol (PEG; MW 4000) and 5 μCi of $^{45}Ca^{2+}$/ml. The free Mg^{2+} (0 8 mM) and Ca^{2+} (as indicated) concentrations were adjusted as described by Van Heeswijk et al. [23]. The incubations, performed at 37°C, were stopped at the indicated times by adding 1 0 ml of ice-cold stop solution containing 150 mM KCl, 5 0 mM $MgCl_2$, 1 0 mM EGTA and 20 mM HEPES/KOH (pH 7 1). The suspension was rapidly filtered (Schleicher and Schüll, GF92). The filters were washed twice with 1 0 ml of ice-cold stop solution, dissolved in scintillation fluid and counted for radioactivity. Total Ca^{2+} was calculated and expressed as nmol/mg of protein Actively stored Ca^{2+} is defined as the difference in total Ca^{2+} retained on the filter after incubation in the presence and absence of ATP IP_3, GTP, GTP-γ-S and heparin were added at the indicated times Protein was determined with a commercial Coomassie Blue kit (Bio-Rad, Richmond, CA, USA) after treatment of the cells with 0.1% Triton X-100

Collagenase was purchased from Cooper Biomedical Inc , Malvern, PA, USA Ruthenium Red was obtained from Merck, Darmstadt, Germany, hyaluronidase, phosphocreatine, creatine kinase, GTP and GTP-γ-S were from Boehringer Mannheim, Germany, NaATP, bovine serum albumin, IP_3, Triton X-100, HEEDTA, nitrilotriacetic acid, EGTA, saponin, phenylmethylsulphonyl fluoride, soya-bean trypsin inhibitor and Trypan Blue were from Sigma, St Louis, MO, USA; HEPES was from Research Organics Inc., Cleveland, OH, USA; heparin was from Organon Teknika, Oss, The Netherlands; polyethylene glycol (PEG; MW 4000) was from Merck, Schuchhardt, Germany. $^{45}Ca^{2+}$ (20 μCi/ml) was purchased from New England Nuclear, Dreieich, Germany. All other reagents were of reagent grade.

Results

Effects of polyethylene glycol on Ca^{2+} uptake

We have shown before that in the presence of 20 μM Ruthenium Red, an inhibitor of mitochondrial Ca^{2+} uptake, permeabilized pancreatic acinar cells rapidly accumulate Ca^{2+} in an energy-dependent pool [11, 12] At a free Ca^{2+} concentration of 0.1 μM and in the presence of 3% (w/v) polyethylene glycol (PEG), ATP-dependent Ca^{2+} uptake reached a steady state of 3 3 nmol of Ca^{2+}/mg of acinar protein (SEM ± 0 2, n = 14) at 10 min and remained unchanged for the next 5–6 min (Fig 1). ATP- dependent Ca^{2+} uptake in the presence of PEG was 112% (SEM ± 7, n = 8) of the value obtained in the absence of PEG.

Effects of IP_3

We have previously shown that IP_3 releases Ca^{2+} from permeabilized pancreatic acinar cells with an EC_{50} of 0.5 μM [12]. Addition of a close to maximal concentration of 1 0 μM IP_3 (Fig. 5A) resulted in a rapid release of 42 1% (SEM ± 2 7, n = 8) of the ATP-dependent pool (Fig 1) In the absence of PEG, IP_3 (1 μM) released 44.5% (SEM ± 2.3, n = 8), indicating that the membrane fusogen did not affect the size of the IP_3-sensitive Ca^{2+} pool. We have previously shown that the IP_3 effect was maximal within 30 s following addition [11]. Figure 1 shows that PEG did not influence the kinetics of IP_3-stimulated Ca^{2+} release No re-uptake of Ca^{2+} was observed for at least 2 min following addition of a submaximal concentration of 0 3 μM IP_3 (Fig. 2) When the IP_3 receptor

antagonist heparin (150 IU/ml) was added 1 min following stimulation with 1.0 μM IP_3, the Ca^{2+} content re-increased from 53% to 84% of the pre-stimulatory value (Table 1). Addition of heparin (150 IU/ml) 1 min before IP_3 inhibited, completely, the IP_3-induced Ca^{2+} release (Table 1). These results indicate that Ca^{2+} re-uptake was prevented

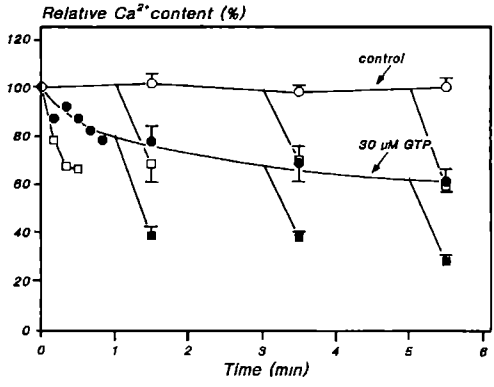

Fig. 1 Time dependence of IP_3- and GTP-induced release of actively stored Ca^{2+} from permeabilized rabbit pancreatic acinar cells

Isolated rabbit pancreatic acinar cells, permeabilized by saponin treatment, were resuspended (4 mg of protein/ml) in a Ca^{2+}-uptake medium (pH 7.1) to which 20 μM Ruthenium Red was added, and were kept on ice for 1 h before use. Ca^{2+} uptake was started by adding 10 μl of permeabilized cells to 90 μl of Ca^{2+}-uptake medium to which 0 or 1.0 mM NaATP was added, and which contained, in addition, 3% PEG. Incubations were carried out at 37°C at a free Ca^{2+} concentration of 0.1 μM, including 5 μCi/ml $^{45}Ca^{2+}$. The free Mg^{2+} concentration was set at 0.8 mM. At 10 min, either saline (open symbols) or GTP at a concentration of 30 μM (closed symbols) was added. IP_3 at a concentration of 1.0 μM (squares) was added at 10, 11, 13 and 15 min. At the indicated times, the reaction mixture was quenched in 1.0 ml of ice-cold stop solution and the suspension was rapidly filtered. $^{45}Ca^{2+}$ retained on the filter after rinsing was determined by liquid scintillation counting. Total Ca^{2+} retained on the filter was calculated and expressed in nmol/mg of acinar protein. Energy-dependent Ca^{2+} uptake is defined as the difference in total Ca^{2+} retained on the filter after incubation in the presence and absence of ATP. Actively stored Ca^{2+} at 10 min is set at 100% to which the other values are related. Permeabilized pancreatic acinar cells accumulated an average of 3.3 nmol of Ca^{2+}/mg of acinar protein (SEM ± 0.2, n = 14). Where indicated the values presented are the means ± SEM of three experiments.

Table 1 Inhibition by heparin of IP_3-induced release of actively stored Ca^{2+} from permeabilized rabbit pancreatic acinar cells

Addition at 10 min	*Addition at 11 min*	*Ca^{2+} content at 12 min (% of saline-treated control)*
Saline	saline	100%
Saline	heparin	109%
Saline	IP_3	63%
Heparin	IP_3	112%
IP_3	saline	53%
IP_3	heparin	84%

Permeabilized rabbit pancreatic acinar cells were loaded with Ca^{2+} in the absence and presence of 1.0 mM NaATP for 10 min at 37°C. Saline, heparin (150 IU/ml) and IP_3 (1.0 μM) were added at the indicated times, and at 12 min the reaction was stopped. Actively stored Ca^{2+} was calculated as described in the caption to Figure 1. The Ca^{2+} content of the saline-treated controls at 12 min is set at 100% to which the other values are related. Data presented are the means of triplicate determinations in a single experiment.

by IP_3 binding to its receptor. Therefore, it can be concluded that at the cell density used in these experiments (0.4 mg of acinar protein/ml), IP_3, at the concentrations examined, is not metabolized within 2 min following addition.

Effects of GTP

In the absence of PEG, the size of the ATP-dependent Ca^{2+} pool remained unchanged following addition of 30 μM GTP (97.5%, SEM ± 4.4, n = 8 as compared to the unstimulated control). However, when PEG (3%) was included in the uptake medium, GTP was capable of releasing maximally 50% of the sequestered Ca^{2+} within 2 min following addition (Fig. 3). The effect of GTP on the ATP-dependent Ca^{2+} pool was clearly dose-dependent. Half-maximal and maximal (39.8%, SEM ± 3.4, n = 16) release occurred at 1.0 μM and 30 μM GTP, respectively. It should be noted, however, that the maximal effect of GTP greatly depended on the cell preparation used (*see* also Fig. 6).

Kinetically, the effects of GTP and IP_3 were different. IP_3 reached its maximal effect within 30 s following addition (discussed above). GTP (30 μM) evoked initially a rapid release of part of the accumulated Ca^{2+} followed by a slower release (Figs 1 & 2).

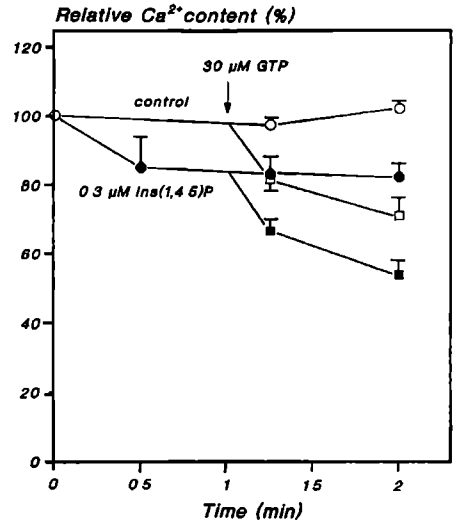

Fig. 2 Time dependence of IP_3- and GTP induced release of actively stored Ca^{2+} from permeabilized rabbit pancreatic acinar cells

Permeabilized rabbit pancreatic acinar cells were incubated in the presence and absence of 1 0 mM NaATP as described in the caption to Figure 1 At 10 min, either saline (open symbols) or IP_3 at a concentration of 0 3 μM (closed symbols) was added GTP at a concentration of 30 μM (squares) was added at 1 1 min The incubation was stopped at the times indicated Actively stored Ca^{2+} was determined as described in the caption to Figure 1 The Ca^{2+} content of the permeabilized cells at 10 min is set at 100% to which the other values are related Data presented are the means ± SEM of three experiments

The effect of GTP (30 μM) on the ATP-dependent Ca^{2+} pool was inhibited, dose-dependently, by GTP-γ-S, a non-hydrolysable GTP analogue (Fig. 4) Inhibition was complete at 100 μM GTP-γ-S. GTP-γ-S (100 μM) alone had virtually no effect on the ATP-dependent Ca^{2+} pool (94.3%, SEM ± 1.2, n = 5, as compared to the control). Moreover, GTP-γ-S (100 μM) did not affect the response to 1.0 μM IP_3 (48.6%, SEM ± 5.3, n = 4 for the combination, compared to 42.1%, SEM ± 2.7, n = 8 for IP_3 alone).

Combinations of GTP and IP_3

In the absence of PEG, the IP_3-sensitive Ca^{2+} pool was not influenced by GTP; Ca^{2+} release in the combined presence of IP_3 (1.0 μM) and GTP (30 μM) was not different from that obtained with 1.0 μM IP_3 alone (42 7%, SEM ± 0.4, n = 3 for the combination, compared to 44.5%, SEM ± 2.3, n = 8 for IP_3 alone).

The experiments described above demonstrate that the effects of GTP on the energy-dependent Ca^{2+} store depend on the presence of PEG. For this reason PEG was included in the Ca^{2+}-uptake medium used in the experiments described below. Addition of IP_3 (1.0 μM) to permeabilized cells, in which part of the ATP-dependent Ca^{2+} pool was already released by GTP (30 μM), did not result in a diminished response to IP_3 (Fig 1) This observation strongly suggests that the effects of IP_3 and GTP on the ATP-dependent Ca^{2+} pool are additive and provides evidence that GTP and IP_3 release

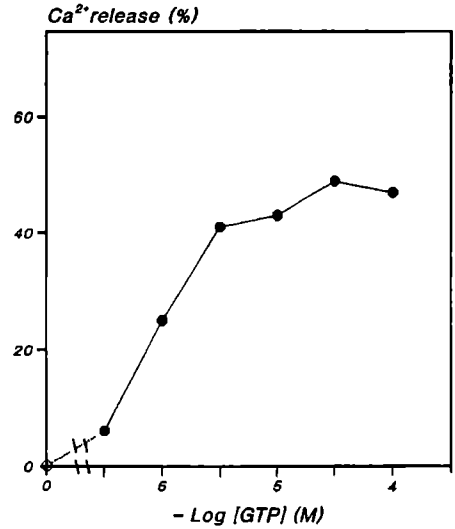

Fig. 3 Dose-response curve for GTP-induced release of actively stored Ca^{2+} from permeabilized rabbit pancreatic acinar cells

Permeabilized rabbit pancreatic acinar cells were loaded with Ca^{2+} in the presence and absence of 1 0 mM NaATP as described in the caption to Figure 1 At 10 min, GTP was added at the indicated concentrations and 2 min later the reaction was stopped Actively stored Ca^{2+} was calculated as described in the caption to Figure 1 The Ca^{2+} content of the saline-treated controls at 12 min is set at 100% to which the other release values are related Data presented are the means of triplicate determinations in a single experiment representative of two

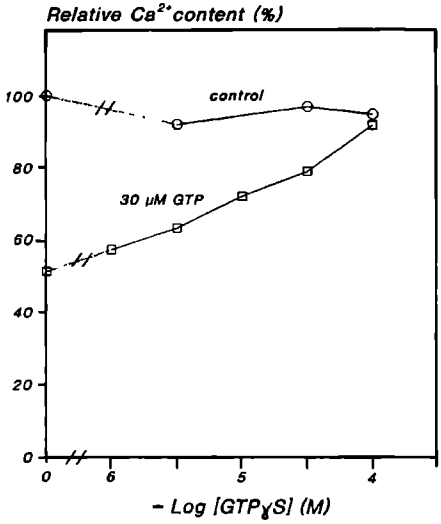

Fig. 4 Dose-inhibition curve for GTP-γ-S on GTP-induced release of actively stored Ca^{2+} from permeabilized rabbit pancreatic acinar cells

Permeabilized rabbit pancreatic acinar cells were loaded with Ca^{2+} in the presence and absence of 1.0 mM NaATP as described in the caption to Figure 1. At 10 min, GTP-γ-S was added at the indicated concentrations. After 30 s, either saline (circles) or GTP at a concentration of 30 μM (squares) was added, and the reaction was stopped 90 s following the addition of GTP. Each concentration was tested in triplicate. Actively stored Ca^{2+} was calculated as described in the caption to Figure 1. The Ca^{2+} content of the saline-treated controls at 12 min is set at 100% to which the other release values are related. Data presented are the means ± SEM of four experiments.

Ca^{2+} from separate pools. The additive effect of GTP (30 μM) on IP_3-induced Ca^{2+} release was observed at all IP_3 concentrations tested (Fig. 5A). Also, when maximal release was obtained with GTP (10–100 μM), IP_3 at a concentration of 1.0 μM gave a further release of the energy-dependent pool (Fig. 5B), indicating that both agents act on different pools. The data presented do not support the idea that GTP, when added to permeabilized pancreatic acinar cells, acts by enlarging the IP_3-releasable Ca^{2+} pool. In addition, Figure 5 shows that, throughout the experiments, the release values obtained with either GTP (30 μM) or IP_3 (1 μM), expressed as percentage of the ATP-dependent pool, varied considerably. This observation will be discussed below.

Effects of ambient free Ca^{2+}

We have previously shown that release of the ATP-dependent Ca^{2+} pool by IP_3 is inhibited by Ca^{2+} at concentrations beyond 1.0 μM [12]. To investigate the effect of the ambient free Ca^{2+} concentration on the response to GTP alone and in combination with IP_3, permeabilized cells were loaded at 0.1, 1.0 and 2.0 μM free Ca^{2+}. The amount of Ca^{2+} accumulated in the ATP-dependent pool clearly depended on the ambient free Ca^{2+} concentration. At 1.0 μM free Ca^{2+}, the size of the ATP-dependent pool was 145% (SEM ± 6, n = 5) of that at 0.1 μM free Ca^{2+}. At 2.0 μM free Ca^{2+}, the size of the pool was 154% (SEM ± 6, n = 5) of that at 0.1 μM free Ca^{2+}, indicating that no further

Table 2 Ca^{2+}-dependence of IP_3- and GTP-induced release of actively stored Ca^{2+} from permeabilized rabbit pancreatic acinar cells

Addition at 10 min	*Addition at 11.5 min*		*Ca^{2+} release (%)*			
		$[Ca^{2+}]_{free}$	*0.1*	*1.0*	*2.0*	*(μM)*
Saline	IP_3		100	100 ± 13	27 ± 1	
GTP (1 μM)	saline		78 ± 19	94 ± 20	89 ± 17	
GTP (30 mM)	saline		117 ± 15	150 ± 25	134 ± 21	
GTP (1 μM))	IP_3		165 ± 17	173 ± 23	119 ± 15	
GTP (30 μM)	IP_3		190 ± 16	227 ± 24	157 ± 21	

Permeabilized rabbit pancreatic acinar cells were loaded with Ca^{2+} in the absence and presence of 1.0 mM NaATP for 10 min at 37°C. Incubations were carried out in the presence of 3% PEG and with the indicated free Ca^{2+} concentrations, including 5 μCi of $^{45}Ca^{2+}$/ml. At 10 min, either saline or GTP (at the indicated concentrations) was added. After 90 s, either saline or IP_3 (1.0 μM) was added, and the reaction was stopped 30 s later. Each combination was tested in triplicate. Actively stored Ca^{2+} was determined as described in the caption to Figure 1. The release value obtained with 1.0 μM IP_3 at 0.1 μM free Ca^{2+} is set at 100% to which the other release values are related. Data presented are the means ± SEM of five experiments.

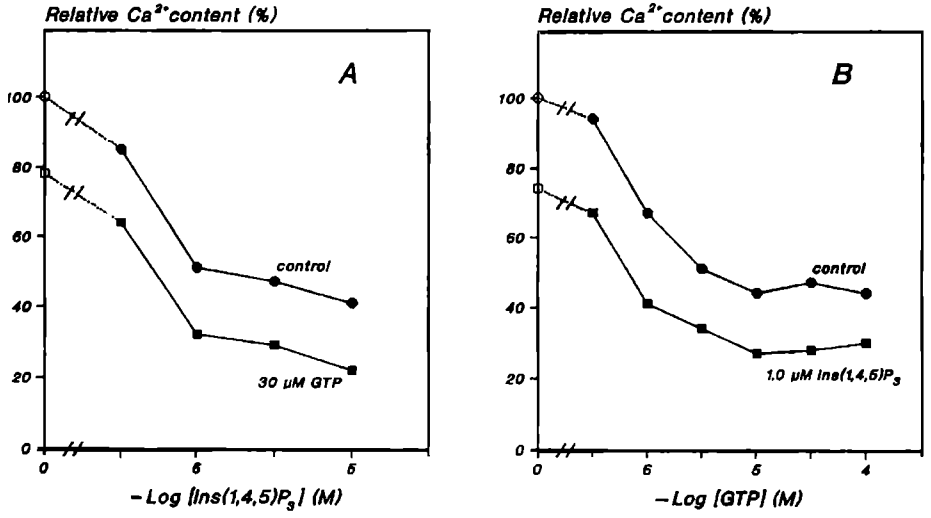

Fig. 5 Additive effects of GTP and IP_3 on the release of actively stored Ca^{2+} from permeabilized rabbit pancreatic acinar cells
Permeabilized rabbit pancreatic acinar cells were loaded with Ca^{2+} in the absence and presence of 1.0 mM NaATP as described in the caption to Figure 1. At 10 min, either saline (circles) or 30 µM GTP (squares) was added. After 30 s, either saline (open symbols) or GTP (closed symbols) was added to both the saline- and the GTP-treated cells. The reaction was stopped at 12 min (A). In a second experiment, either saline (circles) or 1 0 µM IP_3 (squares) was added at 10 min After 30 s, either saline (open symbols) or GTP (closed symbols) was added to both the saline- and the IP_3-treated cells. The reaction was stopped at 12 min (B). Actively stored Ca^{2+} was calculated as described in the caption to Figure 1 The Ca^{2+} content of the saline-treated controls at 12 min is set at 100% to which the other values are related. Data presented are the means of triplicate determinations in a single experiment (B) or the means of triplicate determinations in a single experiment representative of two (A)

increase had occurred. In each experiment the amount of Ca^{2+} released by 1.0 µM IP_3 at 0.1 µM free Ca^{2+} was set at 100% to which all other values were related (Table 2). The amount of Ca^{2+} released by IP_3 at 0.1 and 1.0 µM free Ca^{2+} was virtually the same, indicating that a 1.5-fold increase in size of the ATP-dependent pool was not paralleled by an increased amount of Ca^{2+} to be released by IP_3. At 2.0 µM free Ca^{2+}, the response to IP_3 was significantly (73%) inhibited. In contrast to the findings with IP_3, GTP-induced release of the ATP-dependent Ca^{2+} pool increased rather than decreased at higher ambient free Ca^{2+}, indicating that the GTP-dependent release mechanism is not inhibited by Ca^{2+}. At all three ambient free Ca^{2+} concentrations, the release value obtained with the combination of IP_3 and GTP was virtually the same as the sum of the individual responses. Therefore, the reduced response to IP_3 in combination with GTP at 2.0 µM free Ca^{2+} is apparently completely due to the inhibitory effect of Ca^{2+} on IP_3-induced Ca^{2+} mobilization.

Inversed relationship in size between the GTP- and IP_3-releasable pool

At a free Ca^{2+} concentration of 0.1 µM, permeabilized pancreatic acinar cells accumulated an average of 3.3 nmol of Ca^{2+}/mg of acinar protein in an energy-dependent Ca^{2+} storage pool. Figure 6 shows, however, that throughout the experiments the ATP-dependent Ca^{2+} store varied considerably in size. In spite of this large variation in ATP-dependent Ca^{2+} uptake, the combination of GTP (30 µM) and IP_3 (1 µM) invariably released an average of 73.5% (SD ± 4.6, n = 11) of the energy-dependent store. Linear regression analysis revealed a correlation coefficient of 0.963, indicating that the

amount of Ca^{2+} released by the combined action of GTP and IP_3 was directly proportional to the size of the energy-dependent Ca^{2+} store. In contrast, the Ca^{2+} release values obtained with either GTP or IP_3 alone showed much more variation and correlated less well with the amount of Ca^{2+} accumulated by the energy-dependent store (r values of 0.706 and 0.444, respectively). Relative to the amount of Ca^{2+} released by the combined action of GTP and IP_3, GTP and IP_3 alone released 61.8% (SD ± 15.9, n = 10) and 53.3% (SD ± 13.0, n = 10), respectively. The latter observation suggests that approximately 15% of the amount of Ca^{2+} released by the combined action of GTP and IP_3 might be accumulated in a store sensitive to both GTP and IP_3.

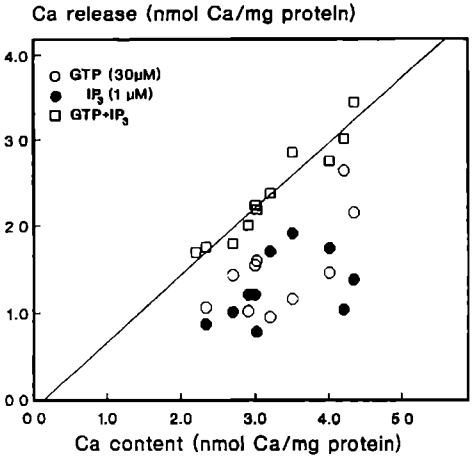

Fig. 6 Relationship between the amount of Ca^{2+} released by GTP and IP_3 alone and in combination, and the size of the energy-dependent Ca^{2+} store in permeabilized rabbit pancreatic acinar cells

Permeabilized rabbit pancreatic acinar cells were loaded with Ca^{2+} in the absence and presence of 1.0 mM NaATP as described in the caption to Figure 1. At 10 min either saline or 30 μM GTP was added. After 30 s, saline was added to both saline-treated cells (unstimulated controls) and GTP-treated cells (open circles). At the same time 1.0 μM IP_3 was added to both saline-treated cells (closed circles) and GTP-treated cells (squares). The reaction was stopped at 12 min. Actively stored Ca^{2+} was calculated as described in the caption to Figure 1 and expressed in nmol/mg of acinar protein. The release values obtained with GTP alone (open circles), IP_3 alone (closed circles), and the combination of GTP and IP_3 (open squares), are plotted against the amount of Ca^{2+} accumulated in the energy-dependent store of the unstimulated controls. Data presented are the means of triplicate determinations of 10 and 11 isolated acinar cell preparations

The data obtained with the close to maximal concentration of 1 μM IP_3 were not different from those obtained with a maximal concentration of 10 μM IP_3. Thus, the combination of 100 μM GTP and 10 μM IP_3 released 77.4% (SD ± 3.7, n = 3) of the energy-dependent Ca^{2+} store, indicating that the combination of 30 μM GTP and 1 μM IP_3 was sufficient to maximally release both pools. Moreover, relative to the amount of Ca^{2+} released by the combination of GTP and IP_3, GTP (100 μM) and IP_3 (10 μM) alone released 42.5% (SD ± 3.6, n = 3) and 58.5% (SD ± 19, n = 3), respectively, values comparable to those obtained with 30 μM GTP and 1.0 μM IP_3.

The possibility of a quantitative relationship between the GTP- and the IP_3-releasable Ca^{2+} store was analysed by plotting the absolute release values obtained with GTP alone as a function of the absolute release values obtained with IP_3 alone (Fig. 7A). However, it is obvious that for a meaningful correlation the variability in the amount of Ca^{2+} released by the combined action of GTP and IP_3 has to be taken into account. Figure 7A shows that in those preparations in which an average of 3.0 nmol of Ca^{2+}/mg of acinar protein (SD ± 0.3, n = 4, open circles) was released by the combined action of GTP and IP_3, the size of the GTP-releasable pool was inversely related with the size of the IP_3-releasable pool (r = –0.998). Also in those preparations in which the combination of GTP and IP_3 released an average of 2.1 nmol of Ca^{2+}/mg of acinar protein (SD ± 0.2, n = 6, closed circles), both pools were inversely related in size (r = –0.546). The inversed relationship between the GTP- and the IP_3-releasable pool (r = –0.787) was also revealed when the release values obtained with either GTP or IP_3 alone were expressed as percentage of the amount of Ca^{2+} released by the combined action of GTP and IP_3 (Fig. 7B).

Discussion

Several studies using permeabilized cells have shown that GTP can cause direct release of Ca^{2+}

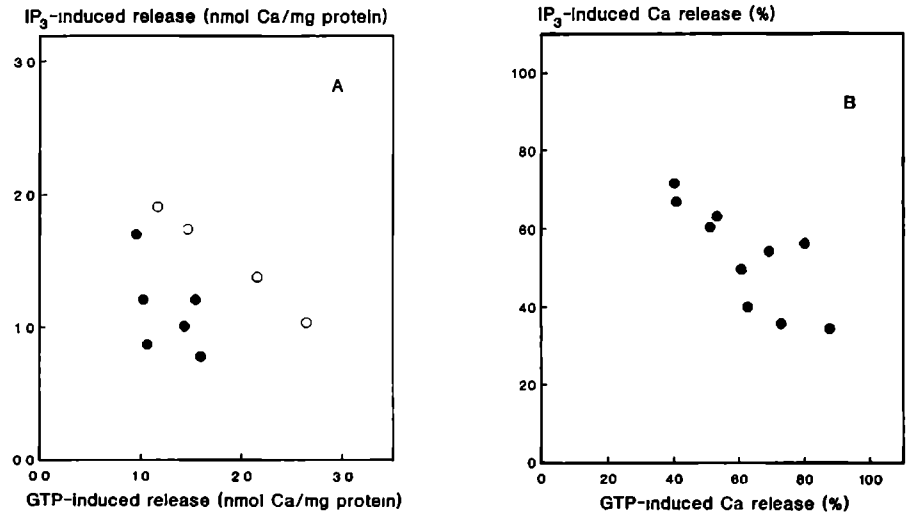

Fig. 7 Inversed relationship in size between the GTP and IP_3-releasable pools in permeabilized rabbit pancreatic acinar cells
The release data shown in Figure 6 are replotted in Figure 7 Figure 7A shows for each individual experiment the release data obtained with GTP alone as a function of the release data obtained with IP_3 alone Based on the amount of Ca^{2+} released by the combined action of GTP and IP_3, the preparations are subdivided into two groups One in which 3 0 nmol Ca^{2+}/mg protein (SD ± 0 3, n = 4, open circles) was released and a second one in which 2 1 nmol Ca^{2+}/mg acinar protein (SD ± 0 2, n = 6, closed circles) was released Linear regression analysis revealed correlation coefficients of –0 998 (open circles) and –0 546 (closed circles), indicating an inversed relationship in size between both pools Figure 7B shows for each individual experiment the relative release value obtained with IP_3 alone as a function of the relative release value obtained with GTP alone The release values are expressed as a percentage of the absolute amount of Ca^{2+} released by the combined action of GTP and IP_3 in the same experiment. Linear regression analysis revealed a correlation coefficient of –0 787

from an energy-dependent storage pool and that this direct GTP-effect occurs in the absence of IP_3 (for a review, *see* [16]) In addition to its direct effect, GTP has also been reported to potentiate the IP_3-induced release of actively stored Ca^{2+} [20] The latter (indirect) effect of GTP clearly involves the IP_3-operated Ca^{2+} channel, whereas the former (direct) effect of GTP does not.

In contrast to earlier studies [24], we now show that GTP can cause direct release of Ca^{2+} from permeabilized pancreatic acinar cells. GTP-induced Ca^{2+} release, however, completely depended on the presence of the fusogen polyethylene glycol (PEG). In this respect, the pancreatic acinar cell differs from other cell types in which a small but significant GTP-effect was already observed in the absence of any fusogen [25–27]. So far, such a strict dependence on PEG has only been reported for GTP-induced Ca^{2+} release from subcellular fractions (for a review, *see* [16])

The fusogen-dependence described above has led to the hypothesis that GTP-induced membrane fusion might be involved in the process of GTP-induced Ca^{2+} mobilization [28–30] Recently, Hampe et al [31] demonstrated GTP-induced and PEG-dependent fusion of pancreatic acinar cell microsomes. However, acidification due to the action of the vacuolar type H^+-pump seemed to be a prerequisite. According to Schulz et al. [10], this H^+-pump is confined to the IP_3-releasable Ca^{2+} pool where it is involved in the process of Ca^{2+} uptake (for a review, *see* [32]). We now show, however, that the amount of Ca^{2+} released from permeabilized acinar cells in response to IP_3 remains unaltered upon the addition of GTP and PEG, indicating that GTP neither enlarges nor releases the IP_3-releasable

pool. This observation suggests that GTP-induced fusion of IP_3-releasable organelles does not underlie the direct GTP-effect observed in the present study.

Recently, we [12] and others [24] reported that IP_3-induced Ca^{2+} release from permeabilized pancreatic acinar cells can be inhibited by Ca^{2+}. In the present study, we show that inhibition of the IP_3-effect by ambient free Ca^{2+} is not paralleled by inhibition of GTP-induced Ca^{2+} release. Therefore, additional evidence is provided that the IP_3-operated Ca^{2+} channel is not involved in GTP-induced Ca^{2+} release from permeabilized acinar cells. A similar Ca^{2+}-insensitivity of the GTP-effect has been described in permeabilized N1E-115 neuroblastoma cells [25]. The physiological meaning of this Ca^{2+}-insensitivity might be that, at elevated free Ca^{2+} levels, Ca^{2+} is transported from the GTP-sensitive pool to the IP_3-releasable pool in a GTP-dependent manner, thereby refilling rather than enlarging [25] the latter one. Such a model might also explain the recent finding that GTP abolishes the apparent desensitization of the Ca^{2+} release observed during repeated IP_3 applications to digitonin permeabilized rat pancreatic acinar cells [24]. In the latter study, the release of Ca^{2+} was measured by means of a Ca^{2+}-selective electrode in a medium not buffered for Ca^{2+}, thus allowing ambient free Ca^{2+} to rise upon the addition of IP_3. Sequestration of part of the Ca^{2+} released through the action of IP_3 in the GTP-sensitive pool could explain the apparent desensitization of the IP_3 response and its reversal by GTP. In addition, the present study shows that GTP releases Ca^{2+} from permeabilized pancreatic acinar cells at a much slower rate than IP_3. In view of this finding, the absence of a direct effect of GTP in the experiments performed by Engling and co-workers might be explained by the immediate re-uptake into the IP_3-releasable store of the Ca^{2+} slowly released by the action of GTP.

Interestingly, both in our study and in the study mentioned above, the GTP-effect was abolished by GTP-γ-S, whereas GTP-γ-S has no effect on its own. This might suggest that, *de facto*, the same phenomenon is studied, but under different experimental conditions.

Compared to permeabilized acinar cells loaded at an ambient free Ca^{2+} concentration of 0.1 μM, the amount of Ca^{2+} released by the action of GTP seemed to be increased in cells loaded at 1 and 2 μM free Ca^{2+}. Whether this reflects increased Ca^{2+} uptake by the GTP-sensitive store or stimulation by Ca^{2+} of the GTP-induced release process needs further investigation.

IP_3 still releases Ca^{2+} from permeabilized acinar cells maximally stimulated with GTP. This additivity and the fact that the IP_3-operated Ca^{2+} channel is apparently not involved in GTP-induced Ca^{2+} release, indicates that both agents act on separate pools. Additive effects of GTP and IP_3 have also been reported for other cell types [26, 33–35]. On the other hand, in permeabilized N1E-115 neuroblastoma cells, a smaller IP_3-releasable pool was shown to be part of a larger GTP-sensitive pool [25, 36].

Gill and co-workers [17, 37], proposed a model for GTP-induced Ca^{2+} release involving junctional connections either between intact compartments or between an intact compartment and non-enclosed (plasma) membrane surfaces. According to this model, the direct GTP-effect observed in the present study could be explained by a PEG/GTP-dependent re-establishment of junctional connections interrupted during, or at the time of, permeabilization. The fact that, in the present study, the addition of GTP in the presence of PEG does not lead to an increase in size of the IP_3-releasable pool, suggests that in permeabilized acinar cells junctional connections are re-established between GTP-sensitive organelles and non-enclosed membrane surfaces rather than between GTP-sensitive and IP_3-releasable organelles. The fact that GTP does not reduce the size of the IP_3-releasable pool indicates that the latter pool is not coupled in a PEG/GTP-dependent manner to non-enclosed membrane surfaces.

Interestingly, the release data, obtained with the different preparations used in the present study, seem to reveal an inversed relationship between the size of the GTP-releasable pool, measured 2 min following addition of GTP, and the size of the IP_3 releasable pool. One explanation might be that the relative size of the IP_3-releasable pool depends on the extent of coupling of the GTP-sensitive pool to the IP_3-releasable pool at the moment of permeabilization. If so, this means that, in the intact cell, GTP activates Ca^{2+} transfer by promoting the

formation of relatively stable junctional connections, not disrupted during permeabilization. Moreover, this observation suggests that once uncoupled from the IP_3-releasable pool the GTP-sensitive pool does not re-couple to the former one in the permeabilized cell. Finally, this observation suggests that if coupled to IP_3-releasable organelles, GTP-sensitive organelles do not couple in a PEG/GTP-dependent manner to non-enclosed membrane surfaces.

In the above context, it is interesting that inclusion of GTP-γ-S in the permeabilization medium led to a significant reduction in size of the IP_3-releasable pool in permeabilized hepatocytes [20]. The effect of GTP-γ-S was explained by assuming that, in the intact cell, terminal GTP-hydrolysis is involved in dynamically coupling GTP-sensitive to IP_3-releasable organelles. Inhibition by GTP-γ-S is a common feature of the GTP-induced Ca^{2+} release [25, 28, 38]. Evidence for the reversibility of the GTP-effect has also been provided by Chueh et al. [36] and by Engling et al. [24].

The present findings are in line with the hypothesis that, in the intact cell, GTP-sensitive organelles, emptied in a PEG/GTP-dependent manner in the permeabilized cell, can be coupled to a greater or lesser extent to IP_3-releasable organelles depending on the physiological state of the cell. Recently, it has been speculated that low-M_r GTP binding proteins may be involved in the process of GTP-mediated communication between GTP-sensitive and Ins(1,4,5)P_3-sensitive pools (for a review, *see* [39]). Identification of the low-M_r GTP binding proteins involved might give an answer to the morphological and functional relationships between the GTP-sensitive pool and the IP_3-releasable pool and/or plasma membrane.

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Received 17 April 1991
Revised 26 June 1991
Accepted 27 June 1991

Chapter 3

Ruthenium red selectively depletes inositol 1,4,5-trisphosphate-sensitive calcium stores in permeabilized rabbit pancreatic acinar cells

In: Journal of Membrane Biology, 135, 153 - 163 (1993)

J Membrane Biol 135 153–163 (1993)

The Journal of
Membrane
Biology

Ruthenium Red Selectively Depletes Inositol 1,4,5-Trisphosphate-Sensitive Calcium Stores in Permeabilized Rabbit Pancreatic Acinar Cells

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Received 8 December 1992/Revised 13 April 1993

Abstract. Rabbit pancreatic acinar cells, permeabilized by saponin treatment rapidly accumulated 3.5 nmol of Ca^{2+}/mg protein in an energy-dependent pool when incubated at an ambient free Ca^{2+} concentration of 100 nM. Maximal loading of the internal stores was reached at 10 min and remained unchanged thereafter. Complete inhibition of the Ca^{2+} pump with thapsigargin revealed that this plateau was the result of a steady-state between slow Ca^{2+} efflux and ATP driven Ca^{2+} uptake. Sixty percent of the pool could be released by Ins(1,4,5)P_3, whereas GTP released another twenty percent. The striking finding of this study is that the energy-dependent store could also be released by ruthenium red. Uptake experiments in the presence of ruthenium red revealed that the dye, at concentrations below 100 μM, selectively reduced the size of the Ins(1,4,5)P_3-releasable pool. Ruthenium red had no effect on the half-maximal stimulatory concentration of Ins(1,4,5)P_3. At concentrations beyond 100 μM the dye also affected the GTP-releasable pool. Comparison with thapsigargin revealed that ruthenium red released Ca^{2+} from stores loaded to steady-state at a rate markedly faster than can be explained by inhibition of the ATPase alone. From the data presented, we concluded that ruthenium red selectively releases Ca^{2+} from the Ins(1,4,5)P_3-sensitive store by activating a Ca^{2+} release channel, whereas Ca^{2+} release from the GTP-sensitive store is predominantly caused by inhibition of the Ca^{2+} pump. The postulated ruthenium red-sensitive Ca^{2+} release channel might be similar to the ryanodine-receptor in muscle.

Key words: Pancreatic acinar cell — Permeabilized acinar cell — Ins(1,4,5)P_3-sensitive Ca^{2+} store — GTP sensitive Ca^{2+} store — ATP-dependent Ca^{2+} uptake — Ruthenium red

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Introduction

Pancreatic acinar cells display an agonist-specific pattern of oscillatory changes in free cytosolic calcium concentration, $[Ca^{2+}]_i$, when stimulated with cholecystokinin (CCK) or acetylcholine (Ach) [28, 37, 45, 46]. Digital imaging microscopy of Fura-2-loaded acinar cells has revealed that Ach evokes a rapid increase in $[Ca^{2+}]_i$ at the edge of the apical pole, followed by a more gradual spread of the $[Ca^{2+}]_i$ rise towards the basal pole [16]. Similar to the situation in adrenal chromaffin cells [4], this spatiotemporal feature of the Ca^{2+} signal has been explained by successive release of Ca^{2+} from Ins(1,4,5)P_3-sensitive stores located immediately beneath the cell membrane [30], possibly at the apical side [16], and from stores possessing a Ca^{2+} release channel activated by a rise of the ambient free Ca^{2+} concentration and thought to be distributed throughout the cytoplasm [16, 30].

The concept of Ca^{2+}-induced Ca^{2+} release (CICR) is adapted from striated muscle, where a depolarization-dependent Ca^{2+} influx is followed by a Ca^{2+}-activated Ca^{2+} release from the sarcoplasmic reticulum through channels sensitive to both ryanodine and caffeine [8, 18, 29]. But, whereas the existence of an Ins(1,4,5)P_3-sensitive Ca^{2+} store in pancreatic acinar cells is well documented [32, 35], evidence for the presence of CICR in this cell type thus far mainly comes from microfluorimetric measurements, which show that short-lasting repetitive Ca^{2+} transients, induced by internal application of Ins(1,4,5)P_3, are markedly broadened by caffeine, indicating that the drug facilitates propagation of the Ca^{2+} wave [28, 30]. In addition, the presence of

caffeine-sensitive Ca^{2+} channels was demonstrated in membrane patches excised from fused acinar cell endoplasmic reticulum vesicles [31] These channels, however, were shown to be voltage dependent and insensitive to Ca^{2+} and ryanodine, indicating that they are not identical to the sarcoplasmic reticulum channel On the other hand, ruthenium red, which inhibits the Ca^{2+}-activated Ca^{2+} channel in muscle [34], did reduce Ca^{2+} channel activity Finally, a caffeine-sensitive pool, insensitive to vanadate and Ins(1,4,5)P_3 but sensitive to ruthenium red, was shown to be present in a pancreatic endoplasmic reticulum vesicle preparation [6]

Permeabilized acinar cells have been used extensively to characterize internal Ca^{2+} stores in more detail [9, 27, 40, 44] We have previously shown that permeabilized acinar cells contain three, nonmitochondrial Ca^{2+} storage pools (i) an Ins(1,4,5)P_3-sensitive pool, (ii) a GTP-sensitive pool, and (iii) a pool insensitive to Ins(1,4,5)P_3 and GTP [40] Thus far, however, we (F H M M van de Put, J J H H M De Pont, and P H G M Willems, *unpublished observations*) and others [6] have been unable to demonstrate a caffeine effect in this preparation

In sarcoplasmic reticulum, Mg^{2+} and ATP have been shown to, respectively, inhibit and stimulate the Ca^{2+}-activated Ca^{2+} channel [22, 23] This leaves the possibility that in permeabilized acinar cells the caffeine effect could have been blocked by Mg^{2+}, whereas loading of a caffeine-sensitive store could have been prevented by ATP, keeping the Ca^{2+}-activated Ca^{2+} channel in a continuous open state The polycationic dye, ruthenium red, is widely used as an effective inhibitor of mitochondrial Ca^{2+} uptake [3, 26] In addition, the dye has been reported to inhibit CICR [18] and, at higher concentrations, Ca^{2+}-ATPase activity [1, 15, 33, 38] In the present study, we have used ruthenium red as a blocker of Ca^{2+}-activated Ca^{2+} channels in an attempt to unmask such channels if hidden by the action of ATP Unexpectedly, however, ruthenium red was found to decrease rather than increase the uptake capacity of the energy-dependent store

The data presented show that ruthenium red can cause the selective reduction in Ca^{2+} uptake capacity of the Ins(1,4,5)P_3-sensitive store by increasing its permeability for Ca^{2+}

Materials and Methods

Pancreatic Acinar Cells

Rabbit pancreatic acinar cells were prepared according to the method of Amsterdam and Jamieson [2] as previously described [40]

Permeabilization of Acinar Cells

Isolated pancreatic acinar cells were washed twice resuspended in a high K medium (1 mg of protein/ml) containing (mM) 135 KCl 1 0 $MgCl_2$ 1 2 KH_2PO_4 0 1 phenylmethyl sulfonyl fluoride and 0 2 mg soya bean trypsin inhibitor per ml and 10 mM HEPES adjusted to pH 7 4 with KOH The cells were permeabilized with saponin (30 μg/ml) for 10 min at 25°C The permeabilized cells were washed and resuspended in ice-cold high-K^+ medium Less than 25% of the cells excluded trypan blue

Ca^{2+} Uptake and Release Experiments

Permeabilized acinar cells were washed twice and resuspended in ice cold Ca^{2+} uptake medium (4 mg of protein/ml) containing (mM) 120 KCl 1 0 $MgCl_2$ 1 2 KH_2PO_4 5 pyruvate 5 succinate 0 5 EGTA 0 5 nitrilotriacetic acid 0 5 HEEDTA and 0 2 mg of soya bean trypsin inhibitor per ml and 20 mM HEPES adjusted to pH 7 1 with KOH The permeabilized cells were kept on ice for maximally 2 hr until use Ca^{2+} uptake was started by adding 10 μl of permeabilized cells to 90 μl of Ca^{2+} uptake medium which contained in addition 10 mM creatine phosphate 10 units of creatine kinase per ml 0 or 1 mM NaATP 3% (w/v) polyethylene glycol (PEG MW 4 000) and 5 μCi of ^{45}Ca per ml The free Mg^{2+} (0 8 mM) and Ca^{2+} (0 1 μM) concentrations were adjusted as described by Van Heeswijk et al [41] The incubations performed at 37°C were stopped at the indicated times by adding 1 0 ml of ice cold stop solution containing (mM) 150 KCl 5 0 $MgCl_2$ 1 0 EGTA and 20 HEPES/KOH (pH 7 1) The suspension was rapidly filtered (Schleicher and Schull GF92 Dassel Germany) The filters were washed twice with 1 0 ml of ice cold stop solution dissolved in scintillation fluid and counted for radioactivity Total Ca^{2+} was calculated and expressed as nmol/mg of protein Actively stored Ca^{2+} is defined as the difference in total Ca^{2+} retained on the filter after incubation in the presence and the absence of ATP Protein was determined with a commercial Coomassie Blue kit (Bio Rad Richmond CA) after treatment of the cells with 0 1% Triton X 100

In another type of experiment an aliquot of the permeabilized cell suspension was diluted ten times in prewarmed Ca^{2+} uptake medium At the indicated times 100 μl of the medium was withdrawn and added to 1 000 μl of ice cold stop solution Actively stored Ca^{2+} was determined using the above filtration procedure

Ca^{2+} Exchange Experiments

ATP dependent Ca^{2+} uptake reaches a plateau level within 10 min [43] This plateau is the result of a steady state between slow Ca^{2+} efflux and ATP driven Ca^{2+} uptake To determine the steady state Ca^{2+} exchange activity a tracer amount of $^{45}Ca^{2+}$ was added to permeabilized cells loaded with $^{40}Ca^{2+}$ for 9 5 min At 9 5 min either saline thapsigargin, or vanadate was added followed by a tracer amount of $^{45}Ca^{2+}$ at 10 min Samples (100 μl) were withdrawn at the indicated times and the Ca^{2+} content was determined as described above At the end of each incubation Ca^{2+} ionophore A23187 was added to completely release the ATP dependent Ca^{2+} storage pool

Materials

Collagenase was purchased from Cooper Biomedical, Malvern, PA EGTA and ruthenium red[1] were obtained from Merck, Darmstadt, Germany, Vanadium pentoxide was from ICN Pharmaceuticals, Life Sciences Group, Plainview, NY, hyaluronidase, phosphocreatine, creatine kinase, A23187 and GTP were from Boehringer, Mannheim, Germany, NaATP bovine serum albumin, Triton X-100, HEEDTA, nitrilotriacetic acid, saponin, phenylmethyl- sulphonyl fluoride, soya-bean trypsin inhibitor and trypan blue were from Sigma, St Louis, MO, HEPES was from Research Organics, Cleveland, OH, polyethylene glycol (MW 4,000) was from Merck, Schuchhardt, Germany, thapsigargin was from LC Services (Sanbio BV, Uden, The Netherlands), $^{45}Ca^{2+}$ (20 μCi/ml) was from New England Nuclear, Dreieich, Germany Ins(1,4,5)P_3 and heparin were generously supplied by Dr P West erduin (Akzo Pharma, Organon Scientific Development Group, Oss, The Netherlands) All other chemicals were of analytical grade

Results

Effect of Ruthenium Red on ATP-Dependent Ca^{2+} Uptake

Permeabilized acinar cells rapidly accumulated Ca^{2+} in an ATP-dependent nonmitochondrial Ca^{2+} store when incubated at an ambient free Ca^{2+} concentration of 0.1 μM (Fig. 1*A*). Uptake reached a steady-state of 3.52 nmol of Ca^{2+}/mg protein (SEM ± 0.38, n = 12) at about 10 min and remained virtually unchanged for the next 5 min. The vesicular nature of the energy-dependent Ca^{2+} store was demonstrated by its complete release following addition of the Ca^{2+} ionophore, A23187 (1 μM). In the presence of 20 μM ruthenium red, ATP-dependent Ca^{2+} uptake was markedly reduced. The effect of the dye on Ca^{2+} uptake was dose dependent and half-maximal, and maximal inhibition occurred at 40 and 300 μM ruthenium red, respectively (Fig. 1*B*) Ruthenium red maximally reduced the steady-state Ca^{2+}-uptake level by 80%, indicating that 20% of the energy-dependent Ca^{2+} storage pool is insensitive to the dye. In the presence of 5 μM ruthenium red, a concentration sufficient to completely inhibit mitochondrial Ca^{2+} uptake at higher ambient free Ca^{2+} concentrations, steady-state Ca^{2+} uptake was not different from the control value, indicating that mitochondria do not accumulate Ca^{2+} at 0.1 μM free Ca^{2+}.

Effect of Ruthenium Red on the Ins(1,4,5)P_3-Sensitive Ca^{2+} Pool

Ins(1,4,5)P_3, when added at a maximally effective concentration of 10 μM, rapidly released 57% (SEM ± 3, n = 12) of the Ca^{2+} stored under steady-state conditions (Table and Fig. 1). Interestingly, the residual Ca^{2+} content after stimulation with Ins(1,4,5)P_3 remained virtually unchanged in permeabilized acinar cells in which the steady-state Ca^{2+}-uptake level was reduced by the action of 20–100 μM ruthenium red (*see also*, Figs. 1 and 2*A*) This observation clearly demonstrates that ruthenium red at concentrations below 100 μM selectively reduces the size of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool. The concentration at which the effect of the dye was half-maximal was estimated to be 30 μM (Fig. 2*B*) Complete depletion of the Ins(1,4,5)P_3-sensitive store was obtained with 300 μM ruthenium red At concentrations beyond 100 μM, however, the dye also reduced the size of the Ins(1,4,5)P_3-insensitive store (Fig 2*A*). Ruthenium red did not change the affinity of the Ins(1,4,5)P_3 receptor for its ligand Both in the absence and presence of 20 or 50 μM ruthenium red Ins(1,4,5)P_3 released Ca^{2} with an EC_{50} of 0 9 μM. Moreover, ruthenium red did not displace radiolabeled Ins(1,4,5)P_3 in a competitive binding assay using the binding protein isolated from adrenal cortical tissue (F H M M van de Put, M van Mackelenbergh, J.J.H H.M De Pont, and P.H G M. Willems, *unpublished observations*)

Effect of Ruthenium Red on the GTP-Sensitive Ca^{2+} Pool

In a previous study we have shown that GTP can cause direct release of Ca^{2+} from permeabilized pancreatic acinar cells, provided that the fusogen polyethylene glycol is present in the incubation medium [40]. GTP, when added at a maximally effective concentration of 100 μM, released 21% (SEM ± 3, n = 12) of the Ca^{2+} stored under steady-state conditions Figure 2*A* shows that the amount of Ca^{2+} released by GTP was not affected by ruthenium red at concentrations below 100 μM At higher concentrations, however, the dye also reduced the size of the GTP-sensitive store. The concentration at which ruthenium red half-maximally reduced the size of the GTP-sensitive store was 400 μM (Fig. 2*B*) We have previously shown that the effects of Ins(1,4,5)P_3 and GTP are additive [40], and in the present study 75%

[1] Ruthenium red is originally described as a hexavalent polycationic dye ($Cl_6H_{42}N_{14}O_2Ru_3$) However, in the present study the tetravalent form ($Cl_4H_{23}N_7O_2Ru_2$) was used, which has been shown to share the same properties with its hexavalent counterpart, in that it completely inhibits mitochondrial Ca^{2+} uptake at 5 μM (this study) and markedly reduces caffeine-induced Ca^{2+} release at 10 μM [6]

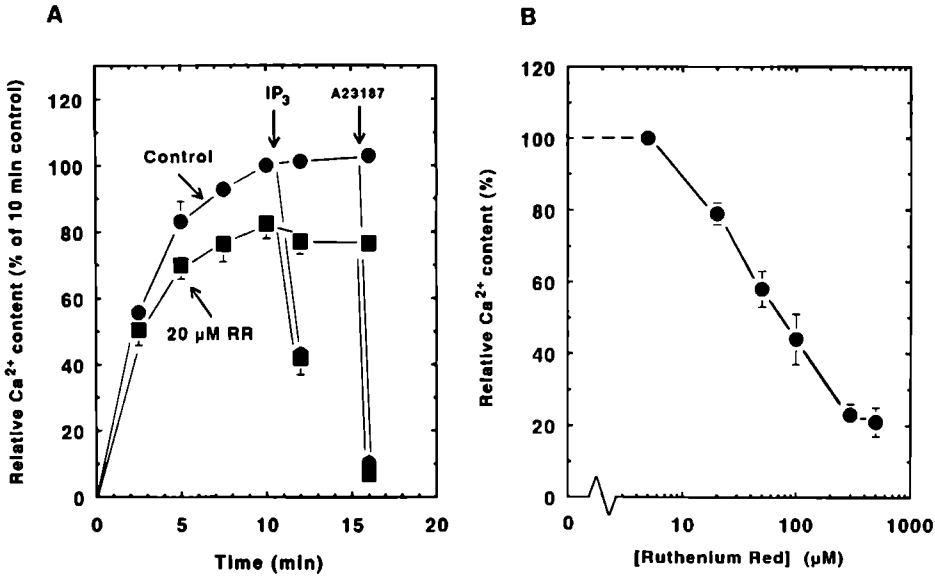

Fig. 1 Inhibitory effect of ruthenium red on Ca^{2+} uptake by nonmitochondrial Ca^{2+} pools in permeabilized pancreatic acinar cells (*A*) Time dependence of ATP dependent Ca^{2+} uptake in the absence and presence of ruthenium red (RR 20 μM) The reaction mixtures were quenched at the indicated times Ins(1 4 5)P_3 (IP_3) and A23187 were added at 10 and 1 0 μM respectively The values presented are not corrected for the uptake obtained in the absence of ATP The Ca^{2+} content in saline treated cells at 10 min is set at 100% to which the other values are related (*B*) Dose dependence of the inhibitory effect of ruthenium red on ATP dependent Ca^{2+} uptake Permeabilized acinar cells were loaded in the presence of the indicated concentrations of ruthenium red and the reaction was quenched at 12 min The values presented are corrected for the uptake obtained in the absence of ATP and the amount of actively stored Ca^{2+} in saline treated cells is set at 100% to which the other values are related The values presented are the means ± SEM of at least three different experiments each performed in triplicate

Table. Selective depletion of the Ins(1 4 5)P_3 sensitive Ca^{2+} pool by ruthenium red in permeabilized pancreatic acinar cells

Loading condition	Residual Ca content (%)	
Ruthenium Red (μM)	Saline	Ins(1 4 5)P_3 (10 μM)
Saline	100%	43 ± 3%
20 μM RR	77 ± 4%	42 ± 5%
50 μM RR	56 ± 5%	39 ± 5%
100 μM RR	47 ± 6%	38 ± 6%

Isolated rabbit pancreatic acinar cells permeabilized by saponin treatment were loaded with Ca^{2+} to steady state in the absence and presence of the indicated concentrations of ruthenium red (RR) At 10 5 min either saline or Ins(1 4 5)P_3 (10 μM) was added and 1 5 min later the reaction was stopped Actively stored Ca^{2+} in saline treated cells is set at 100% to which the other values are related Saline treated permeabilized cells actively accumulated an average of 3 5 nmol of Ca^{2+} /mg of acinar protein (SEM ± 0 4 n = 12) The values presented are the means ± SEM of at least three different experiments each performed in triplicate

(SEM ± 2, n = 8) of actively stored Ca^{2+} was released by the combined action of Ins(1,4,5)P_3 and GTP Figure 2*A* shows that ruthenium red only slightly affected the Ins(1,4,5)P_3 and GTP- insensitive store

Effects of Thapsigargin and Vanadate on ATP-Dependent Ca^{2+} Uptake

In order to test the possibility that ruthenium red acts by inhibiting Ca^{2+} ATPase activity, the effects of the dye on ATP-dependent Ca^{2+} uptake were compared with those of vanadate and thapsigargin Figure 3 shows that ATP-dependent Ca^{2+} uptake was blocked almost completely in the presence of 0 5 mM vanadate or 1 μM thapsigargin By contrast, ruthenium red maximally reduced ATP-dependent Ca^{2+} uptake by 80% (Fig 1*B*), indicating that the dye does not fully mimic the effect of these well-known inhibitors of Ca^{2+}-ATPase activity

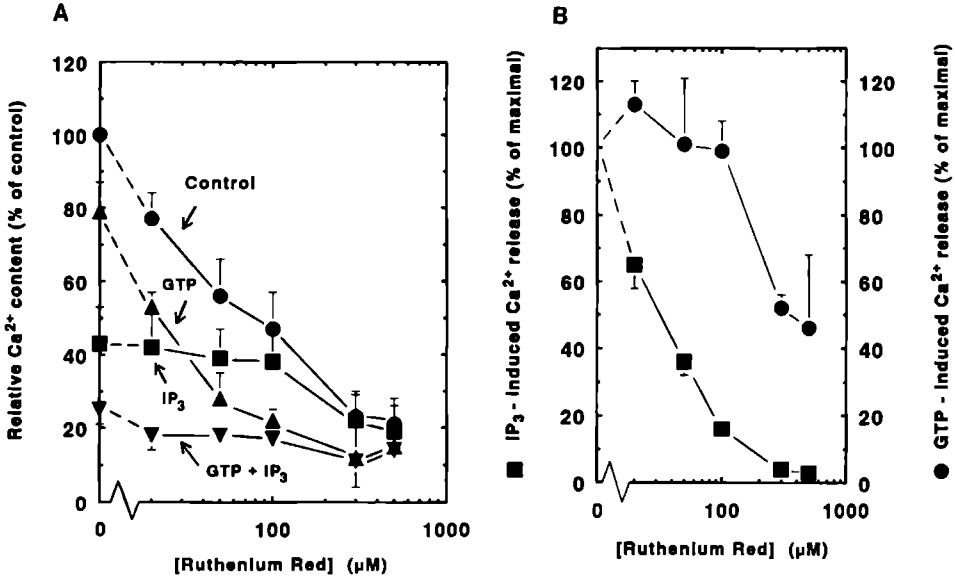

Fig 2 Dose dependence of the inhibitory effect of ruthenium red on Ca uptake by Ins(1 4 5)P_3 and GTP sensitive Ca storage organelles in permeabilized pancreatic acinar cells Permeabilized acinar cells were loaded with Ca^{2} in the absence and presence of the indicated concentrations of ruthenium red (RR) At 10 min either saline or GTP (100 μM) was added At 10 5 min either saline or Ins(1 4 5)P_3 (10 μM) was added to both the saline treated controls and the cells prestimulated with GTP The incubation mixtures were quenched at 12 min (*A*) Residual Ca content following stimulation with either saline (control) GTP (100 μM) Ins(1 4 5)P_3 (10 μM) or the combination of GTP and Ins(1 4 5)P_3 as a function of the ruthenium red concentration in the uptake medium Actively stored Ca^{2} in saline treated cells loaded in the absence of ruthenium red is set at 100% to which the other values are related (*B*) Relative sizes of the Ins(1 4 5)P_3- and GTP sensitive Ca storage pools as a function of the ruthenium red concentration in the uptake medium In each experiment the release value obtained with either 100 μM GTP (21% SEM ± 3 n = 12) or 10 μM Ins(1 4 5)P_3 (57% SEM ± 3 n = 12) in permeabilized cells loaded in the absence of ruthenium red is set at 100% to which the other values are related The values presented are the means ± SEM of at least three different experiments determined in triplicate in each experiment

Effect of Thapsigargin on the Ins(1,4,5)P_3-Sensitive Ca^{2+} Pool

Loading of permeabilized pancreatic acinar cells in the presence of thapsigargin resulted in a dose-dependent reduction of the steady-state Ca^{2+} uptake level The IC_{50} for the effect of thapsigargin on ATP-dependent Ca^{2+} uptake was calculated to be 3 5 nM Loading in the presence of thapsigargin resulted in a dose-dependent decrease of the residual Ca^{2+} content obtained after maximal stimulation with Ins(1,4,5)P_3 This suggests that, in contrast to ruthenium red, thapsigargin does not selectively reduce the size of the Ins(1,4,5)P_3-sensitive pool

Effects of Ruthenium Red, Vanadate and Thapsigargin on the Steady-State Ca^{2+} Level

Addition of 10 μM Ins(1,4,5)P_3 to permeabilized pancreatic acinar cells loaded with Ca^{2+} to steady-state led to a rapid and complete release of Ca^{2+} from the Ins(1,4,5)P_3-sensitive store (Fig 4*A*) No reuptake of Ca^{2+} was observed during the next four minutes, indicating that in the continuous presence of Ins(1,4,5)P_3 a lower steady state is established Addition of heparin (150 U/ml) resulted in a time-dependent refilling of the Ins(1,4,5)P_3 sensitive store Figure 4*B* shows that vanadate (0 5 mM), thapsigargin (1 μM) or ruthenium red (500 μM) induced a time-dependent decrease of the amount of actively stored Ca^{2+} However, the kinetics with which these compounds released Ca^{2+} from the energy-dependent store differed considerably Vanadate was less effective than thapsigargin, whereas ruthenium red released actively stored Ca^{2+} at a markedly faster rate than thapsigargin The effect of ruthenium red was clearly dose dependent (Fig 4*C*) Following addition of ruthenium red a lower steady-state was established Subsequent addition of heparin resulted in a rapid, time-dependent reuptake of Ca^{2+} (*not shown*) The latter observation clearly demonstrates the re-

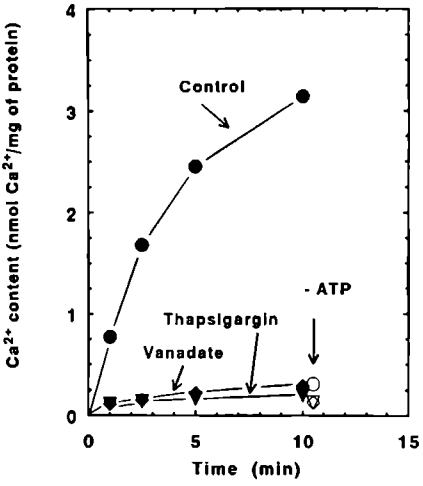

Fig. 3 Effects of vanadate and thapsigargin on ATP dependent Ca^{2+} uptake in permeabilized pancreatic acinar cells Permeabilized acinar cells were loaded with Ca^{2+} in the absence (control) and presence of either vanadate (0 5 mM) or thapsigargin (1 0 μM) Ca^{2+} uptake was started by adding 50 μl of permeabilized acinar cells to 450 μl of Ca^{2+} uptake medium to which 0 (open symbols) or 1 0 mM NaATP (filled symbols) was added At the indicated times 100 μl aliquots were quenched in 1 0 ml ice cold stop solution and the Ca^{2+} content was determined The data presented are from a single experiment

versible nature of the effect of ruthenium red on the energy-dependent Ca^{2+} storage pool

Effects of Ruthenium Red, Vanadate and Thapsigargin on Ca^{2+} Exchange

In order to confirm that under conditions of steady-state loading the inhibitory effect of thapsigargin on the Ca^{2+} pump was indeed complete, permeabilized acinar cells were loaded with Ca^{2+} in the absence of $^{45}Ca^{2+}$ At 10 min, $^{45}Ca^{2+}$ was added to study the rate of Ca^{2+} exchange under steady-state conditions Actively stored Ca^{2+} was rapidly exchanged and Fig 5 shows that the exchange was almost completely blocked by 1 μM thapsigargin Surprisingly, 0 5 mM vanadate only slightly inhibited the exchange of actively stored Ca^{2+} From the observation that thapsigargin blocked almost completely the exchange of Ca^{2+} under conditions similar to those at which ruthenium red released actively stored Ca^{2+} at a much faster rate than thapsigargin, it can be concluded that ruthenium red does not act by inhibition of Ca^{2+}-ATPase activity alone

Similar to thapsigargin, ruthenium red effectively inhibited the exchange process (Fig 6*A*) The effect of the drug was clearly dose dependent and 500 μM ruthenium red was as effective as 1 μM thapsigargin Depletion of the Ins(1,4,5)P_3-sensitive pool immediately before the addition of $^{45}Ca^{2+}$ markedly decreased the size of the exchangeable pool (Fig 6*B*) The remainder of the exchangeable pool was virtually similar in size to that obtained in the presence of 100 μM ruthenium red, indicating that at this concentration the drug selectively affects the Ins(1,4,5)P_3-sensitive pool Addition of heparin following complete depletion of the Ins(1,4,5)P_3-sensitive store markedly increased the size of the exchangeable pool (*data not shown*) However, the selectivity of the inhibitory effect of ruthenium red could not be tested under these conditions due to the antagonizing effect of heparin

Discussion

In recent years, the polycationic dye ruthenium red has been reported to affect a variety of Ca^{2+} translocating processes with different sensitivities [1, 3, 7, 15 26, 33, 38] In the present study, we have investigated the effects of ruthenium red on the process of ATP-dependent Ca^{2+} uptake in permeabilized pancreatic acinar cells to unmask a possible caffeine-sensitive Ca^{2+} store hidden by the stimulatory action of ATP on the caffeine-sensitive Ca^{2+}-activated Ca^{2+} channel, thereby keeping it in a permanent open state and thus preventing the store to be loaded with Ca^{2+} The data presented show that ruthenium red has marked effects on the process of ATP-dependent Ca^{2+} transport in the permeabilized cell system, but that these effects are difficult to interpret in terms of inhibition of a caffeine-sensitive Ca^{2+}-activated Ca^{2+}-release channel

The striking finding of this study is that ruthenium red can cause a marked decrease in steady-state Ca^{2+} content of the Ins(1,4,5)P_3-sensitive Ca^{2+} store without having an effect on other Ca^{2+} storage organelles This observation suggests that the dye acts at different entities Possible sites of action are (i) Ca^{2+} pumps (ii) Ca^{2+} binding proteins, and (iii) Ca^{2+} release channels In the uptake experiments, in which ruthenium red was present from the beginning, the dye dose-dependently reduced the vesicular Ca^{2+} content Similar observations have been reached with smooth muscle microsomes [15] and isolated sarcoplasmic reticulum [11] In both studies, the effect of the dye was explained by inhibition of the ATP-driven Ca^{2+} pump Such an inhibitory action of the dye has been demonstrated for the plasma membrane Ca^{2+} pump [24] and the sarcoplasmic reticulum Ca^{2+} pump [1] However, our

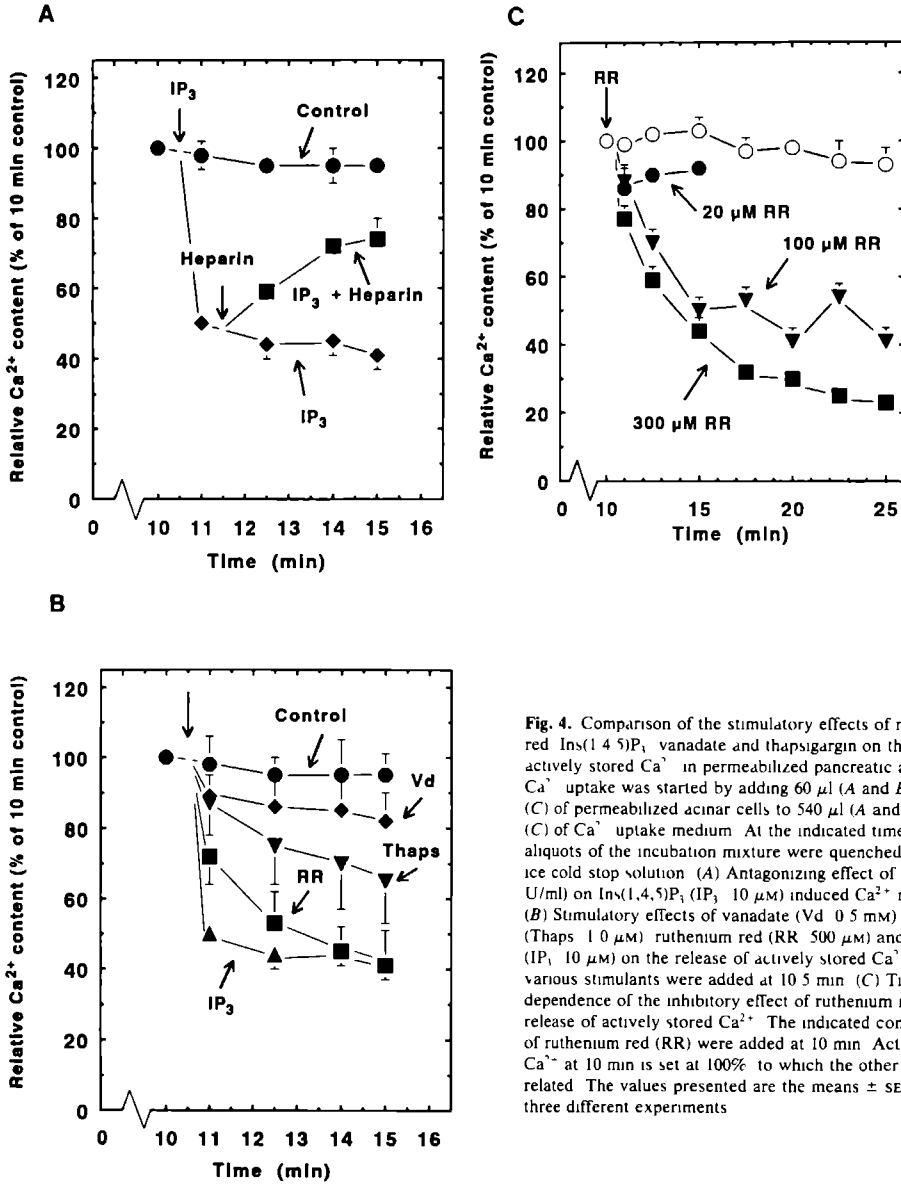

Fig. 4. Comparison of the stimulatory effects of ruthenium red red $Ins(1,4,5)P_3$, vanadate and thapsigargin on the release of actively stored Ca^{2+} in permeabilized pancreatic acinar cells Ca^{2+} uptake was started by adding 60 μl (*A* and *B*) or 90 μl (*C*) of permeabilized acinar cells to 540 μl (*A* and *B*) or 810 μl (*C*) of Ca^{2+} uptake medium At the indicated times 100 μl aliquots of the incubation mixture were quenched in 1 0 ml ice cold stop solution (*A*) Antagonizing effect of heparin (150 U/ml) on $Ins(1,4,5)P_3$ (IP_3 10 μM) induced Ca^{2+} mobilization (*B*) Stimulatory effects of vanadate (Vd 0 5 mM) thapsigargin (Thaps 1 0 μM) ruthenium red (RR 500 μM) and $Ins(1,4,5)P_3$ (IP_3 10 μM) on the release of actively stored Ca^{2+} The various stimulants were added at 10 5 min (*C*) Time and dose dependence of the inhibitory effect of ruthenium red on the release of actively stored Ca^{2+} The indicated concentrations of ruthenium red (RR) were added at 10 min Actively stored Ca^{2+} at 10 min is set at 100% to which the other values are related The values presented are the means ± SEM of at least three different experiments

finding that ruthenium red released Ca^{2+} from steady-state loaded permeabilized cells at a markedly faster rate than thapsigargin, a potent inhibitor of intracellular-type Ca^{2+} pumps [19], or vanadate, indicates that inhibition of Ca^{2+} pump activity alone cannot explain the effects observed

In order to verify that vanadate and thapsigargin inhibited completely the activity of the Ca^{2+}-ATPase under the condition that the internal store was maximally loaded, both agents were tested in an exchange experiment Thapsigargin inhibited completely the ATP-driven Ca^{2+} pump However,

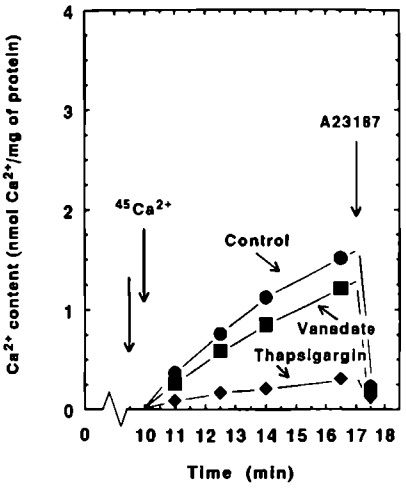

Fig 5. Inhibitory effects of thapsigargin and vanadate on the rate of Ca^{2+} exchange in steady state loaded permeabilized pancreatic acinar cells Permeabilized acinar cells were loaded with Ca^{2+} in the absence of $^{45}Ca^{2+}$ Ca^{2+} uptake was started by adding 60 μl of permeabilized acinar cells to 540 μl of Ca^{2+} uptake medium At 9 5 min either vanadate (0 5 mM) or thapsigargin (1 0 μM) was added, followed at 10 min by a tracer amount of $^{45}Ca^{2+}$ At the indicated times 100 μl aliquots of the incubation mixture were quenched and the Ca^{2+} content was determined At 17 min 1 0 μM A23187 was added to determine the amount of actively stored Ca^{2+}

compared to the uptake experiment, vanadate was less effective in the exchange experiment Similar discrepancies have been reported before, and it has been suggested that at high intravesicular Ca^{2+} concentrations the low affinity Ca^{2+} binding site of the Ca^{2+} transport ATPase is occupied with Ca^{2+}, a condition known to abolish the inhibitory effect of vanadate [21, 33]

A second explanation for the inhibitory effect of ruthenium red on the accumulation of Ca^{2+} in internal stores has been provided by Vale and Carvalho [38] They suggested that the dye might act by displacing Ca^{2+} bound to the Ca^{2+} binding protein, calsequestrin, which is present in the lumen of the sarcoplasmic reticulum Such a displacement has been shown for purified calsequestrin [5] However, the possibility that ruthenium red acts this way is rather unlikely since the positively charged molecule is not expected to simply pass the vesicular membrane In this context, it has been shown that in intact muscle fibers the plasma membrane is rather impermeable to ruthenium red [48]

Interestingly, however, recent studies have provided evidence that the binding capacity of intraluminal Ca^{2+} storage proteins, such as calsequestrin, can be modulated by agents interacting with specific proteins located at the vesicular membrane In muscle, the localization of the Ca^{2+} binding protein has been shown to be confined to the terminal cisternae of the sarcoplasmic reticulum, where the process of Ca^{2+}-activated Ca^{2+} release takes place Detailed morphological studies have shown that the Ca^{2+} storage protein, calsequestrin, forms a network in the center of the terminal cisternae and that this network is anchored to the sarcolemma [10] In addition, biochemical studies have provided evidence that calsequestrin is, either directly or indirectly, associated with the ryanodine receptor [17, 25] Finally, the ryanodine receptor has been demonstrated to be the caffeine-sensitive Ca^{2+}-activated Ca^{2+} channel of the sarcoplasmic reticulum [18] Recently, experimental evidence has shown that caffeine interacts with the ryanodine receptor to stimulate the dissociation of Ca^{2+} bound to calsequestrin [12, 13] Thus, receptor-mediated opening of the Ca^{2+} channel is paralleled by receptor-mediated mobilization of intraluminally bound Ca^{2+}

In view of the present study, the observation that ruthenium red can bind to the ryanodine receptor might be of interest [5] The present study shows that ruthenium red can cause the rapid release of Ca^{2+} from the $Ins(1,4,5)P_3$-sensitive store Although a similar observation has not been reported in muscle, it is attractive to speculate that in nonmuscle cells ruthenium red interacts with a specific, ryanodine receptor-like protein to promote the dissociation and subsequent release from the endoplasmic reticulum of Ca^{2+} bound to an intraluminal Ca^{2+} storage protein The presence of such a calsequestrin-like protein in intracellular structures of several nonmuscle cell types, including the pancreatic acinar cell, is well documented [20, 36, 39, 42] However, whether a receptor similar to the ryanodine receptor is present in pancreatic acinar cells is still unclear

In subfractionation studies it was shown that the acinar cell fraction which was most sensitive towards $Ins(1,4,5)P_3$, was also most sensitive towards caffeine [6] This might indicate that the $Ins(1,4,5)P_3$-sensitive pool contains also a caffeine-sensitive Ca^{2+} release channel A similar observation has been reached with PC12 cells [47] Thus, a possible explanation for the observed selective reduction of the $Ins(1,4,5)P_3$-sensitive Ca^{2+} pool is that ruthenium red binds to a specific channel, thereby reducing the binding capacity of the luminal Ca^{2+} binding protein Absence of the postulated, specific Ca^{2+} channel in the GTP-sensitive store would then explain the relative insensitivity of this store to ruthenium red The effect of relatively high ruthenium red concentrations on the Ca^{2+}-accumulating capacity of this compartment might then be

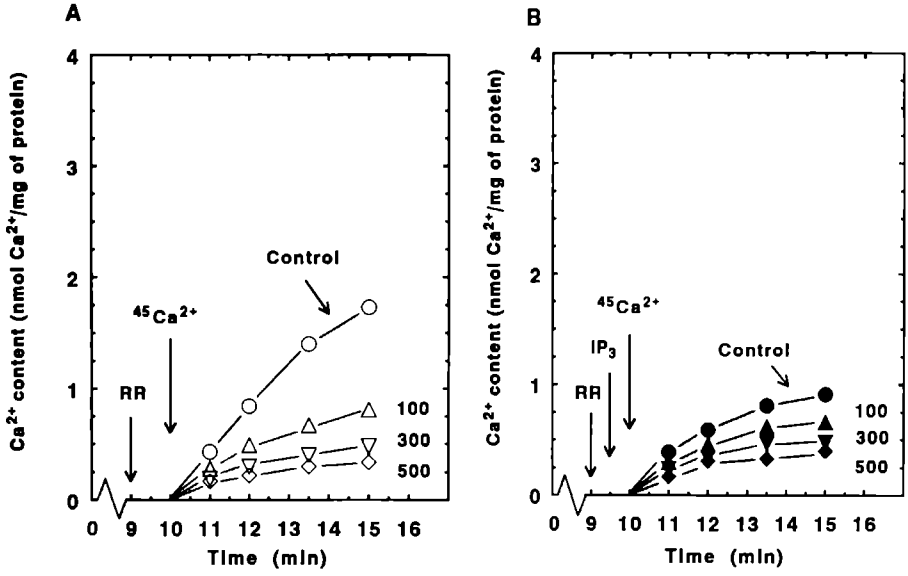

Fig. 6. Dose dependence of the effect of ruthenium red on steady-state Ca^{2+} exchange rates in control and Ins(1,4,5)P_3 prestimulated permeabilized pancreatic acinar cells. Permeabilized acinar cells were loaded with Ca^{2+} in the absence of $^{45}Ca^{2+}$. Ca^{2+} uptake was started by adding 50 μl of permeabilized acinar cells to 450 μl of Ca^{2+}-uptake medium. At 9 min, the indicated concentrations of ruthenium red (RR) were added, followed at 10 min by a tracer amount of $^{45}Ca^{2+}$ (*A*). (*B*) In a second experiment, 10 μM Ins(1,4,5)P_3 (IP_3) was added at 9.5 min, followed at 10 min by a tracer amount of $^{45}Ca^{2+}$. At the indicated times, 100 μl aliquots of the incubation mixture were quenched, and the Ca^{2+} content was determined.

explained by inhibition of the activity of the Ca^{2+}-ATPase of the GTP-sensitive store.

A third possible explanation for the effects of ruthenium red is that the dye acts directly on a Ca^{2+} channel or on a protein that gates it. Addition of ruthenium red to steady-state loaded cells, in which Ca^{2+} efflux from the energy-dependent store was balanced by ATP-driven Ca^{2+} uptake, led to a net efflux of Ca^{2+}. The net efflux rate was considerably higher immediately after addition of the dye than several seconds later. At the long term, however, the net efflux rate decreased either to zero, at concentrations below 100 μM, or to values similar to those obtained with thapsigargin, at concentrations beyond 100 μM. The importance of this observation is that, when given at concentrations below 100 μM, ruthenium red releases Ca^{2+} in a manner identical to Ins(1,4,5)P_3, whereas at higher concentrations this rapid phase is followed by a sustained phase reflecting inhibition of Ca^{2+}-ATPase activity. But, whereas Ins(1,4,5)P_3 interacts with a specific receptor to open Ca^{2+} channels, the mechanism by which ruthenium red evokes the rapid release of Ca^{2+} is still unclear (*see above*). A striking finding is that submaximal concentrations of ruthenium red not only selectively affect the Ins(1,4,5)P_3-releasable pool but also induce the "quantal" type of release characteristic for Ins(1,4,5)P_3 [14, 27]. This might suggest that both ruthenium red and Ins(1,4,5)P_3 act on the same mechanism to release actively stored Ca^{2+}. However, ruthenium red did not interfere with the binding of Ins(1,4,5)P_3 to its receptor.

In conclusion, the data presented show that ruthenium red can cause the selective release of Ca^{2+} from the Ins(1,4,5)P_3-sensitive pool in permeabilized pancreatic acinar cells. The rate with which Ca^{2+} is released is relatively fast and suggests that the dye acts by increasing the open-state probability of a Ca^{2+} release channel, other than the Ins(1,4,5)P_3-operated Ca^{2+} channel. By analogy with the muscle cell, where ruthenium red binds to the ryanodine receptor and where activation of this receptor by caffeine leads to the rapid dissociation of Ca^{2+} bound to calsequestrin, it is speculated that in non-muscle cells, such as the pancreatic acinar cell, ruthenium red binds to a ryanodine receptor-like protein, which, similar to the ryanodine receptor itself, possesses intrinsic channel activity to evoke

the rapid dissociation of Ca^{2+} bound to calreticulin in order to allow the massive release of Ca^{2+} into the cytosol

The research of Dr P H G M Willems has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences

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Chapter 4

Basal Mg^{2+}-dependent ATPase activity of rat liver microsomes is not influenced by ambient free Ca^{2+}

In: European Journal of Biochemistry, 218, 959 - 962 (1993)

Eur J Biochem 215, 959–962 (1993)

Basal Mg^{2+}-dependent ATPase activity of rat liver microsomes is not influenced by ambient free Ca^{2+}

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(Received August 20, 1993) – EJB 93 1266/6

The potent inhibitor of Ca^{2+}-Mg^{2+}-ATPase activity, thapsigargin, has been used to investigate the effect of ambient free Ca^{2+} on basal Mg^{2+}-dependent ATPase activity in rat liver microsomes. Thapsigargin non-competitively inhibited both Ca^{2+}-stimulated ATP-dependent Ca^{2+} uptake and Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activity. At a concentration of 1 μM, thapsigargin completely inhibited the Ca^{2+}-pump activity without affecting Mg^{2+}-dependent ATPase activity measured in the absence of Ca^{2+}. Increasing the ambient free Ca^{2+} concentration did not affect the basal Mg^{2+}-dependent ATPase activity. The data presented indicate that ATPase activity measured in the absence of Ca^{2+} is a reliable measure for the basal Mg^{2+}-dependent ATPase activity and that consequently the Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activity can indeed be defined as the difference between the ATPase activity measured in the presence and the absence of Ca^{2+}.

Organelles of non-mitochondrial origin play an important role in both Ca^{2+} homeostasis and signal transduction. The Ca^{2+}-sequestering function of these organelles is provided for by the Ca^{2+}-transport ATPase. Rat liver microsomes are extensively used to study the characteristics of this Ca^{2+}-translocating enzyme. Ca^{2+} accumulation occurs in an ATP-dependent manner and is stimulated by submicromolar Ca^{2+} concentrations [1]. The Mg^{2+}-dependent and Ca^{2+}-stimulated ATPase activity (Ca^{2+}-ATPase) is considered to represent the activity of a phosphoprotein with a molecular mass of 116–125 kDa [2–4]. A major problem using liver microsomes is the relatively large basal Mg^{2+}-stimulated ATPase activity. It has been suggested that a phosphoprotein of 30 kDa is mainly responsible for the latter activity [4]. More detailed studies using the rather unspecific Ca^{2+}-ATPase inhibitor fluorescein 5′-isothiocyanate have suggested that this basal Mg^{2+}-dependent ATPase activity (Mg^{2+}-ATPase) is inhibited by ambient free Ca^{2+} [5, 6]. Since Ca^{2+}-ATPase activity is defined as the difference between ATP hydrolysis in the absence and presence of Ca^{2+}, this would mean that in studies on the Ca^{2+} dependence of the enzyme the values obtained with higher Ca^{2+} concentrations would be underestimated. This would especially be the case in preparations such as liver microsomes, which contain substantial Mg^{2+}-ATPase activity.

In the present study we have reinvestigated the effects of ambient free Ca^{2+} on the basal Mg^{2+}-ATPase activity using the sesquiterpene lactone thapsigargin, which acts as a specific inhibitor of all members of the family of the sarcoplasmic and endoplasmic reticulum Ca^{2+}-pumps [7]. The data obtained show that ambient free Ca^{2+} has no effect on basal Mg^{2+}-ATPase activity in rat liver microsomes and that therefore Ca^{2+}-ATPase activity can be defined as the difference between the ATPase activity measured in the presence and absence of Ca^{2+}.

MATERIALS AND METHODS

Preparation of rat liver microsomes

Rat liver heavy microsomal fraction was prepared according to Reinhart and Bygrave [8]. Briefly, one rat liver was homogenized in 10 vol. medium containing 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM EGTA and 5 mM Hepes/KOH, pH 7.0. The homogenate was centrifuged at 1000 *g* for 5 min, after which time the supernatant was removed and centrifuged for another 10 min at 8000 *g*. The resulting supernatant was again centrifuged for 20 min at 36000 *g* and the pellet obtained this way was resuspended in medium containing 250 mM sucrose, 1 mM dithiothreitol, 5 mM Hepes/KOH, pH 7.0 and 10 mM KCl. After centrifugation at 36000 *g* for 20 min, the pellet consisting of heavy microsomes was resuspended in the same buffer to a final protein concentration of 5–10 mg/ml. The microsomal suspension was stored on ice until use.

Ca^{2+}-uptake experiments

Ca^{2+} uptake was started by adding 10 μl microsomal suspension to 90 μl warm Ca^{2+} uptake medium containing 150 mM KCl, 0.5 mM EGTA, 0.5 mM nitrilotriacetic acid, 0.5 mM Heedta, 20 mM Hepes/KOH, pH 7.0, 1 mM NaN_3, 5 μCi/ml $^{45}Ca^{2+}$ and 0 mM or 3 mM NaATP. The free Mg^{2+}

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Abbreviations. Ca^{2+}-ATPase, Ca^{2+}-Mg^{2+}-ATPase; Ca^{2+}-stimulated Mg^{2+}-dependent ATPase, Mg^{2+}-ATPase; Mg^{2+}-dependent ATPase, FITC, fluorescein 5′-isothiocyanate.

Enzymes. Ca^{2+}-ATPase (EC 3.6.1.38); Mg^{2+}-ATPase (EC 3.6.1.3).

Note. G. J. Visser deceased on January 16, 1993.

(1 5 mM) and Ca^{2+} (as indicated) concentrations were adjusted as described by Schoenmakers et al [9] The incubations were performed at 37°C After 20 s, the reaction was quenched in 1 ml ice-cold stop solution containing 150 mM KCl 1 mM EGTA and 20 mM Hepes/KOH, pH 7 0 Preliminary experiments revealed that ATP-dependent Ca^{2+} uptake was linear with time during the first 20 s of incubation The suspension was rapidly filtered through a nitrocellulose filter with a pore size of 0 45 µm (Schleicher and Schull) The filters were washed twice with 1 ml ice cold stop solution, dissolved in scintillation fluid and radioactivity determined Total Ca^{2+} was calculated and expressed as nmol Ca^{2} /mg protein Actively stored Ca^{2+} is defined as the difference in total Ca^{2+} retained on the filter after incubation in the presence and absence of ATP The Ca^{2+}-uptake rate is expressed in nmol Ca^{2+} mg protein^{-1} min ^{1}

ATPase assay

The reaction was started by adding 50 µl microsomal suspension to 450 µl warm incubation medium The incubation medium was essentially the same as described above with the exception that 1 µM Ca^{2+} ionophore A23187 was included The incubations were performed at 37°C After 10 min the incubation was quenched by adding 0 5 ml ice-cold 10% (mass/vol) trichloroacetic acid The precipitated protein was pelleted by centrifugation at 1000 *g* for 1 min The phosphate concentration in the supernatant was measured according to the method described by Fiske and SubbaRow [10] The ATPase activity is expressed in nmol P_i mg protein^{-1} min^{-1} The Ca^{2-} ATPase activity is defined as the difference between the activities observed in the presence and absence of the indicated Ca concentration

Protein determination

The protein content was determined with a commercial Coomassie-blue kit (Bio-Rad) with bovine serum albumin (Bio Rad) as a standard

Materials

Thapsigargin was purchased from LC Services Corporation $^{45}Ca^{2}$ (20 mCi/ml) was obtained from New England Nuclear A23187 was from Calbiochem, EGTA was from Merck, NaATP, Heedta and nitrilotriacetic acid were from Sigma, dithiothreitol and Hepes were from Research Organics Inc All other chemicals were of analytical grade

RESULTS AND DISCUSSION

Rat liver microsomes rapidly accumulated Ca^{2} in an ATP dependent manner The rate of the ATP-dependent Ca^{2} uptake increased with increasing ambient free Ca^{2+} concentration (Fig 1 A) A maximum was reached at 1 µM free Ca^{+}, above this concentration the Ca^{2+}-uptake rate tended to decrease Similar biphasic kinetics have been reported by Dawson [1] Double reciprocal (Lineweaver-Burk) plotting of the uptake rate versus the ambient free Ca^{2} concentration revealed a maximal uptake rate of 15 2 nmol Ca^{2} mg protein^{1} min^{-1} The K_m for Ca^{2} was calculated to be 0 42 µM Measurement of the Ca^{2+}-ATPase activity showed a similar dependence on the ambient free Ca^{2} concentration (Fig 2) Maximal stimulation was obtained with 1 µM free

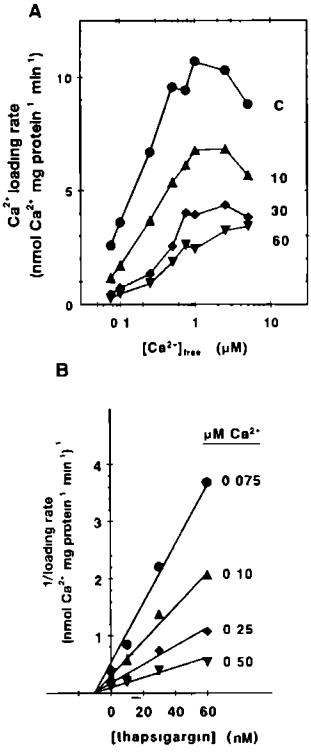

Fig. 1. Effect of thapsigargin on the initial Ca^{2+}-uptake rate in rat liver microsomes. Active Ca^{2} uptake was measured at 1 5 mM free Mg and the indicated free Ca concentrations Incubations were performed at 37°C The reactions were stopped after 20 s The concentrations of thapsigargin were 0 (●) 10 (▲) 30 (◆) and 60 (▼) nM (A) Dose stimulation curve for the effect of Ca on the Ca^{-} uptake rate (B) Dixon plot of the rates obtained at the indicated free Ca concentrations The data presented are the mean of two independent measurements each performed in triplicate The standard error of the triplicates was less than 15%

Ca^{2+}, whereas higher Ca^{2+} concentrations tended to inhibit the activity of the enzyme Lineweaver-Burk analysis revealed a V_{max} of 15 5 nmol P_i mg protein^{-1} min^{-1} and a K_m for Ca^{2+} of 0 29 µM The kinetic parameters obtained are in good agreement with those reported in the literature [1, 2] Using the same rat liver microsome preparation, Dawson and co-workers reported a maximum Ca^{2-} uptake rate of 16 nmol Ca^{2} mg protein^{-1} min^{-1}, as well as a maximum Ca^{2+} ATPase activity of 16 nmol P mg protein^{-1} min^{-1} In both cases a K_m for Ca^{2} of 0 20 µM was reported

Thapsigargin dose-dependently inhibited the rate of ATP-dependent Ca^{2+} uptake without affecting the shape of the dose/response curve for the stimulatory effect of ambient free Ca^{2} (Fig 1A) Dixon analysis revealed a non competitive type of inhibition by thapsigargin (Fig 1 B) The K_i for thapsigargin was determined to be 10 nM The non competitive nature of the inhibitory effect of thapsigargin with respect to stimulation of Ca^{2} uptake by ambient free Ca^{2+} has also

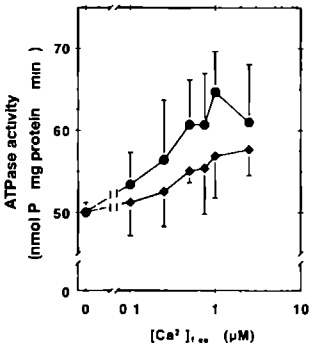

Fig 2 Effect of thapsigargin on the Ca^2 ATPase activity in rat liver microsomes ATPase activity was measured at the indicated free Ca concentrations as described in the legend to Table 1 Thapsigargin was added at 0 nM (●) or 10 nM (◆) The rate of phosphate production is expressed in nmol P produced mg protein min The values presented are normalized to the average of the values obtained in the absence of added Ca The data presented are the mean + SEM of nine and three independent incubations performed in the absence and presence of thapsigargin respectively

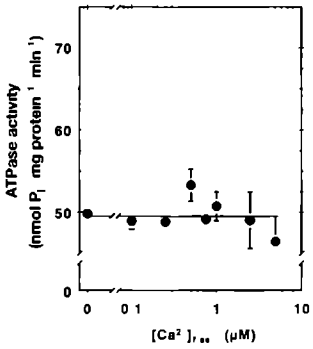

Fig 3 Effect of the free Ca^2 concentration on basal Mg^2 ATPase activity in rat liver microsomes ATPase activity was measured in the presence of 1 μM thapsigargin as described in the legend to Table 1 Incubations were performed at the indicated free Ca concentrations The data presented are the mean ± SEM of six independent experiments each performed in triplicate

Table 1 Effect of thapsigargin on the basal Mg^2 ATPase activity in rat liver microsomes Rat liver microsomes were incubated in the presence of 3 mM NaATP for 10 min at 37°C A23187 (1 μM) was routinely included in the assay medium to prevent Ca accumulation The incubations were performed in the absence of added Ca and at a free Mg concentration of 1 5 mM Thapsigargin was added at the indicated concentrations The values presented are the mean ± SEM of the number of experiments given in parentheses

Thapsigargin	Mg ATPase activity
μM	nmol P min mg protein
0	51 4 ± 4 5 (9)
0 01	45 5 ± 1 1 (3)
0 1	53 6 ± 5 6 (6)
1	49 8 ± 0 9 (9)

been described for the sarcoplasmic reticulum Ca pump [11] At a concentration of 1 μM thapsigargin completely abolished Ca uptake (data not shown)

ATPase activity was studied in the presence of 1 5 mM free Mg and various concentrations of free Ca At nominally free Ca the ATPase activity was 50 nmol P mg protein min A similar value has been reported by Dawson and Fulton [2] Thapsigargin did not influence this basal Mg -ATPase activity (Table 1) This observation is in agreement with the finding of Thastrup et al [12] The ATPase activity increased as a function of the ambient free Ca^2 concentration (Fig 2) At a concentration of 10 nM thapsigargin half maximally inhibited the Ca stimulated part of the ATPase activity The extent of inhibition was similar to that observed in the Ca uptake experiment indicating that ATP hydrolysis is tightly correlated with Ca transport activity

Since 1 μM thapsigargin completely inhibited the ATP dependent Ca uptake without affecting basal Mg ATPase activity this concentration was used to investigate the effects of Ca on basal Mg ATPase activity Fig 3 shows that elevation of the ambient free Ca concentration up to 5 μM did not have any effect on basal Mg ATPase activity This observation is in clear contrast with the findings reported by Kraus Friedman et al [6] In order to inhibit the activity of the Ca ATPase they treated the rat liver microsomes with fluorescein 5 isothiocyanate (FITC) Labeling with FITC requires a basic pH and control experiments revealed that this condition alone reduced the Ca ATPase activity by more than 50% whereas fluorescein 5 isothiocyanate treatment resulted in a further reduction by no more than 25% [6] Thus prior treatment with FITC does not completely inhibit Ca ATPase activity In contrast basal Mg ATPase activity was not affected by basic treatment nor FITC This FITC treated microsomal preparation was used to study the effect of ambient free Ca on basal Mg ATPase activity Basically there are three explanations for the observation that ambient free Ca inhibited the ATPase activity measured under these conditions Firstly Ca inhibits the basal Mg ATPase activity whereby the inhibitory effect has to exceed the stimulatory effect of Ca on the remainder of the Ca ATPase activity Secondly Ca has no effect on the basal Mg ATPase activity but together with Mg it causes FITC to completely inhibit the Ca ATPase activity Thirdly Ca also enables FITC to inhibit the basal Mg ATPase activity Apart from this the possibility exists that the inhibitory effect of basic treatment becomes more pronounced during subsequent incubation in the presence of both Ca and Mg Unfortunately no data were presented on the Ca ATPase activity measured in the presence of Mg with prior treatment of microsomes at basic pH [6] However if in the present study Ca did have an inhibitory effect on basal Mg ATPase activity this inhibitory effect must have been masked by a stimulatory effect on Ca ATPase activity However our Ca uptake data clearly demonstrate that 1 μM thapsigargin completely inhibits the transport activity of the pump whereas the inhibitory effect of FITC was shown to be far from complete [6] Moreover the Ca uptake experiments show that thapsigargin inhibits in a non competitive manner with respect to ambient free Ca

In conclusion, the data presented demonstrate that basal Mg^{2+}-ATPase activity in rat liver microsomes is independent of the ambient free Ca^{2+} concentration, indicating that the ATPase activity measured in the absence of Ca^{2+} is a reliable measure for basal Mg^{2+}-stimulated ATPase activity and that, consequently, the Ca^{2+}-ATPase activity can indeed be defined as the difference between ATPase activity measured in the presence and the absence of Ca^{2+}.

The work of Dr G J Visser was supported by a grant from the Foundation for Biological Research (BION), which is subsidized by the Netherlands Organization for Scientific Research. The research of Dr P H G M Willems has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

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Chapter 5

Induction of Ca^{2+} oscillations by selective, U73122-mediated, depletion of inositol-trisphosphate-sensitive Ca^{2+} stores in rabbit pancreatic acinar cells

In: European Journal of Physiology, 427, 233 - 243 (1994)

Pflugers Arch (1994) 427 233–243

Pflügers Archiv
European Journal of Physiology

Induction of Ca^{2+} oscillations by selective, U73122-mediated, depletion of inositol-trisphosphate-sensitive Ca^{2+} stores in rabbit pancreatic acinar cells

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Received November 11 1993/Received after revision and accepted December 21 1993

Abstract. The effect of the putative inhibitor of phospholipase C activity, U73122, on the Ca^{2+} sequestering and releasing properties of internal Ca^{2+} stores was studied in both permeabilized and intact rabbit pancreatic acinar cells. U73122 dose dependently inhibited ATP-dependent Ca^{2+} uptake in the inositol (1,4,5)-trisphosphate-[Ins(1,4,5)P_3] sensitive, but not the Ins(1,4,5)P_3-insensitive, Ca^{2+} store in acinar cells permeabilized by saponin treatment. In a suspension of intact acinar cells, loaded with the fluorescent Ca^{2+} indicator, Fura-2, U73122 alone evoked a transient increase in average free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{av}$), which was largely independent of external Ca^{2+}. Addition of U73122 to cell suspensions prestimulated with either cholecystokinin octapeptide or JMV-180 revealed an inverse relationship in size between the U73122- and the agonist-evoked $[Ca^{2+}]_{av}$ transient. Moreover, thapsigargin-induced inhibition of intracellular Ca^{2+}-ATPase activity resulted in a $[Ca^{2+}]_{av}$ transient, the size of which was not different following maximal prestimulation with either U73122 or agonist. These observations suggest that U73122 selectively affects the Ins(1,4,5)P_3-sensitive Ca^{2+} store, whereas thapsigargin affects both the Ins(1,4,5)P_3-sensitive and -insensitive Ca^{2+} store. Digital-imaging microscopy of Fura-2-loaded acinar cells demonstrated that U73122, in contrast to thapsigargin, evoked sustained oscillatory changes in $[Ca^{2+}]_i$. The U73122 evoked oscillations were abolished in the absence of external Ca^{2+}. The ability of U73122 to generate external Ca^{2+} dependent Ca^{2+} oscillations suggests that depletion of the agonist-sensitive store leads to an increase in Ca^{2+} permeability of the plasma membrane and that the Ins(1,4,5)P_3-insensitive Ca^{2+} pool is necessary for the Ca^{2+} oscillations.

Key words: Pancreatic acinar cell – Cholecystokinin-octapeptide – JMV 180 – U73122 – Thapsigargin – Ca^{2+} oscillations – Permeabilized acinar cells

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Introduction

Digital-imaging microscopy of Fura-2-loaded pancreatic acinar cells has revealed that receptor activation gives rise to a rapidly spreading Ca^{2+} signal, initiated at the secretory pole [13, 14, 21, 37, 38]. The initial rise in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is independent of external Ca^{2+} [13, 14, 41], indicating that it involves the inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] stimulated release of Ca^{2+} from intracellular stores [2]. Subsequent spreading of the Ca^{2+} signal to the basal pole is believed to take place by a mechanism referred to as Ca^{2+}-induced Ca^{2+} release (CICR) [14, 21, 22, 24, 25, 31, 38, 42, 43]. The concept of CICR is adapted from striated muscle where a depolarization-dependent Ca^{2+} influx is followed by a Ca^{2+}-activated Ca^{2+} release from the sarcoplasmic reticulum through channels sensitive to both ryanodine and caffeine [5, 15, 23]. Recent observations, including the induction of heparin-insensitive cytosolic Ca^{2+} rises by Ca^{2+} infusion [14] and the slowing down of acetylcholine-evoked Ca^{2+} waves by caffeine and ryanodine [21], have provided substantial evidence for the existence of such Ca^{2+}-activated Ca^{2+} release channels in the pancreatic acinar cell. In addition it has been demonstrated that the Ins(1,4,5)P_3 receptor itself can provide for CICR [3, 7, 19] and recent studies have indicated that this mechanism might also be involved in wave propagation in the pancreatic acinar cell [14].

In the continuous presence of cholecystokinin (CCK) or acetylcholine, pancreatic acinar cells display periodic increases in $[Ca^{2+}]_i$. These repetitive Ca^{2+} rises develop similarly to the initial Ca^{2+} transient. Upon lowering of the external Ca^{2+} concentration ongoing Ca^{2+} oscillations evoked by acetylcholine immediately stop, whereas those evoked by CCK gradually extinguish [47]. The latter observation indicates that external Ca^{2+} is not obligatory in inducing receptor-evoked Ca^{2+} oscillations, but is needed for their maintenance. It has been demonstrated that during the second phase of a transient Ca^{2+} rise external Ca^{2+} enters the cell [16], in order to

compensate for the loss of Ca^{2+} during the rising phase of the Ca^{2+} transient [35, 36], and it has been hypothesized that this increase in Ca^{2+} permeability of the plasma membrane is regulated by the state of depletion of the agonist-sensitive intracellular Ca^{2+} store [26, 27, 34]. Although the mechanism by which this occurs is largely unknown [18], studies performed by Bahnson et al. [1] suggest a role for guanosine 3′,5′-cyclic monophosphate (cGMP) as the intracellular messenger in agonist-stimulated Ca^{2+} influx in the pancreatic acinar cell. On the other hand, recent studies have provided evidence for the involvement of a small messenger referred to as "Ca^{2+} influx factor" [28].

In parotid acinar cells, depletion of the agonist-sensitive pool by thapsigargin has been demonstrated to result in oscillatory changes in $[Ca^{2+}]_i$ [10]. Using pancreatic acinar cells, however, Muallem et al. [20] failed to evoke cytosolic Ca^{2+} oscillations by inhibition of intracellular Ca^{2+}-ATPase activity using 2,5-di-(t-butyl)-1,4-benzohydroquinone (BHQ). One explanation might be that in pancreatic acinar cells compounds such as thapsigargin and BHQ inhibit Ca^{2+} uptake by both Ins(1,4,5)P_3-sensitive and Ins(1,4,5)P_3-insensitive intracellular Ca^{2+} stores [40], and that in this cell type the latter store plays an important role in the generation of periodic Ca^{2+} transients. Unravelling this problem requires the availability of specific inhibitors of Ca^{2+} uptake by the Ins(1,4,5)P_3-sensitive pool.

It is generally accepted that Ins(1,4,5)P_3 is the intracellular messenger for the release of Ca^{2+} from intracellular stores [2]. However, recent data suggest that Ins(1,4,5)P_3 is not the only Ca^{2+} mobilizing messenger. In permeabilized rat pancreatic acinar cells the high-affinity CCK receptor agonist JMV-180 [Boc-Tyr(SO^{3-})-Nle-Gly-Trp-Nle-Asp-2-phenylethylester] evoked the release of Ca^{2+} from a Ca^{2+} store insensitive to Ins(1,4,5)P_3 [29]. In addition, the putative inhibitor of phospholipase C activity, the aminosteroid U73122, was found to block completely Ca^{2+} mobilization by CCK octapeptide (CCK_8), but not by JMV-180, in individual rat pancreatic acinar cells [46]. Both observations agree with the idea that JMV-180 mobilizes Ca^{2+} through an intracellular messenger other than Ins(1,4,5)P_3. Recent studies have provided evidence that sphingoid bases may act as intracellular messengers in receptor-mediated Ca^{2+} release from Ins(1,4,5)P_3-sensitive stores. In permeabilized muscle cells [12] and rat pancreatic acinar cells [48] both sphingosine and sphingosylphosphorylcholine were demonstrated to release Ca^{2+} from intracellular stores. In addition, intact Swiss 3T3 cells [50] and intact parotid [33] and rat pancreatic [48] acinar cells were shown to respond to sphingosine [33, 48, 50] and sphingosine-1-phosphate [48] with an increase in $[Ca^{2+}]_i$.

Preliminary experiments with U73122 in isolated rabbit pancreatic acinar cells revealed that this compound not only inhibited Ca^{2+} mobilization in response to both CCK_8 and JMV-180, but also mobilized Ca^{2+} by itself. These discrepancies with earlier reports in literature [46, 48] urged us to investigate the effects of U73122 in more detail. The present study shows that U73122, in addition to its reported inhibitory effect on phospholipase C activity [46], prevented Ca^{2+} accumulation in the Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} store, leaving the Ins(1,4,5)P_3-insensitive store largely intact. Moreover, U73122 was demonstrated to induce external Ca^{2+}-dependent oscillatory changes in $[Ca^{2+}]_i$, which were not observed with thapsigargin, a specific inhibitor of intracellular Ca^{2+} pumps [17]. The occurrence of U73122-evoked Ca^{2+} oscillations may be explained by selective depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} store triggering the influx of Ca^{2+}, which is then accumulated in the Ins(1,4,5)P_3-insensitive store from which it is subsequently released by CICR.

Materials and methods

Materials. Collagenase was purchased from Cooper Biomedical, Malvern, Pa., USA; Fura-2/AM was from Molecular Probes, Eugene, Ore., USA. Hyaluronidase, phosphocreatine and creatine kinase were obtained from Boehringer, Mannheim, Germany; CCK-JMV-180 was from Research Plus, Bayonne, N. J., USA; Ryanodine was from Calbiochem, La Jolla, Calif., USA; Caffeine was from Janssen Pharmaceutica, Beerse, Belgium; CCK_8, TPA, NaATP, bovine serum albumin, Ins(1,4,5)P_3, Triton X-100, *N*-hydroxyethylethylenediaminetriacetic acid (HEEDTA), nitrilotriacetic acid, ethylenebis(oxonitrilo)tetraacetate (EGTA), saponin, phenylmethylsulphonyl fluoride and soybean trypsin inhibitor were from Sigma, St. Louis, Mo., USA; HEPES was from Research Organics, Cleveland, Ohio, USA; polyethylene glycol (mol · wt · 4000) was from Merck, Darmstadt, Germany; thapsigargin was from LC Services Corporation, Woburn, Mass., USA. U73122 and U73343 were generously supplied by Dr. J.E. Bleasdale, The Upjohn Company, Kalamazoo, Mich., USA. D-Lorglumide was a gift from Dr. P.H. M. Hermkens, Medicinal Chemistry II, Organon, Oss, The Netherlands. Heparin was generously supplied by Dr. P. Westerduin, AKZO Pharma, Organon Scientific Development Group, Oss, The Netherlands. $^{45}Ca^{2+}$ (7.4×10^5 Bq/ml) was purchased from New England Nuclear, Dreieich, Germany. All other chemicals were of reagent grade.

Pancreatic acinar cells. Rabbit pancreatic acinar cells were prepared by enzymatic digestion, using collagenase and hyaluronidase, as previously described [44, 45]. At the end of the isolation procedure, the acinar cells were resuspended in a 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid/tris(hydroxymethyl)aminomethane (HEPES/TRIS) medium (pH 7.4) containing 133 mM NaCl, 4.2 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.8 mM glucose, 0.2 mg/ml soybean trypsin inhibitor, an amino acid mixture according to Eagle, 1% (w/v) bovine serum albumin and 10 mM HEPES, adjusted with TRIS to pH 7.4 (loading medium) and kept on ice until use.

Fluorescence measurements in suspensions of acinar cells. An aliquot of acinar cells, at a size sufficient to perform three fluorescence measurements, was removed and resuspended in fresh loading medium. The cells were incubated in the presence of 3 μM Fura-2/AM, added from a dimethyl sulphoxide stock, for 30 min at room temperature. Excess Fura-2/AM was removed by washing twice with HEPES/TRIS medium containing 0.1% (w/v) bovine serum albumin (incubation medium). The Fura-2-loaded acinar cells were kept at room temperature until use. Immediately before measurement one-third of the suspension was centrifuged for 5 min at 100 *g* and resuspended in fresh incubation medium prewarmed to 37°C. The suspension was transferred to a cuvette placed in a Shimadzu RF-5000 spectrofluorophotometer equipped with a magnetic stirrer and a thermostatic cuvette holder. Fluorescence measurements were carried out at 37°C. The fluorescence

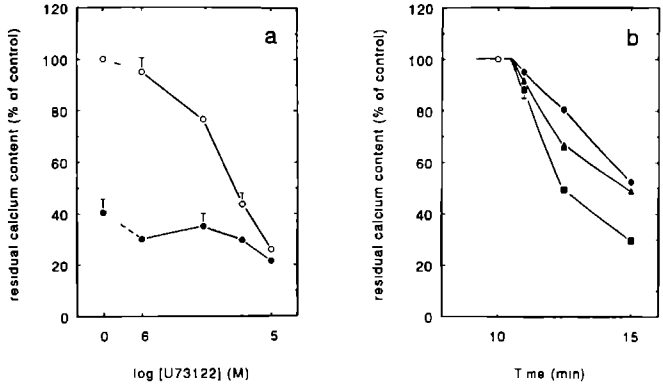

Fig 1 a, b Selective depletion of inositol(1 4 5) trisphosphate [Ins(1 4 5)P_3] sensitive Ca storage organelles in permeabilized pancreatic acinar cells **a** Dose/inhibition curve for U73122 on ATP dependent Ca uptake in Ins(1 4 5)P sensitive Ca storage organelles Permeabilized pancreatic acinar cells were loaded with Ca to steady state in the absence and presence of the indicated concentrations of U73122 The incubations were carried out at 37°C at an ambient free Ca^{2} concentration of 190 nM At 10 5 min either saline (*open circles*) or 10 µM Ins(1 4 5)P (*closed circles*) was added and 1 5 min later the reaction was stopped Actively stored Ca in saline treated cells incubated in the absence of U73122 is set at 100% to which all other values are related In the absence of U73122 permeabilized acinar cells actively accumulated 3 4 nmol Ca^{2} /mg of acinar protein (SFM ± 1 0 $n = 3$) The values presented are the mean ± SFM of 3 independent determinations each of which performed in triplicate In the presence of 10 µM U73343 Ca accumulation reached a steady state value of 96% whereas subsequent stimulation with 10 µM Ins(1 4 5)P decreased the residual Ca content to 29% These observations demonstrate that U73343 did not interfere with ATP dependent Ca accumulation or Ins(1 4 5)P stimulated Ca release **b** Comparison of the effects of U73122 and thapsigargin alone and in combination on the release of actively stored Ca in permeabilized acinar cells After loading with Ca to steady state for 10 5 min permeabilized acinar cells were treated with either 1 µM thapsigargin (*closed circles*) 6 µM U73122 (*closed triangles*) or the combination of thapsigargin and U73122 (*closed squares*) The reaction mixtures were quenched at the indicated times Actively stored Ca at 10 min is set at 100% to which all other values are related Where indicated the data presented are the mean ± SEM of 3 independent determinations The combination was tested in duplicate in a single experiment

emission ratio at 490 nm was monitored after excitation at 340 and 380 nm

Fluorescence measurements in individual acinar cells For fluorescence measurements an aliquot of the chilled suspension was removed centrifuged and resuspended in loading medium The temperature of the suspension was brought to 37°C and the cells were loaded with Fura 2 in the presence of 3 3 µM Fura 2 AM for 15 min After loading excess Fura 2 AM was removed by washing twice with incubation medium The Fura 2 loaded cells were resuspended in 400 µl of the incubation medium and transferred to a thermostatic (32°C) incubation chamber The cells were allowed to attach to a glass cover slip forming the bottom of the chamber for 10 min The chamber was placed on the stage of an inverted microscope and an epifluorescent ×40 magnification oil immersion objective was used to allow simultaneous monitoring of an average of close to 150 acinar cells Dynamic video imaging was carried out as described previously [44] using the MagiCal hardware and Tardis software provided by Joyce Loebl (Dukesway Team Valley Gateshead Tyne and Wear UK) The fluorescence emission ratio at 492 nm was monitored as a measure of [Ca] after excitation at 340 nm and 380 nm

Permeabilization of acinar cells Isolated pancreatic acinar cells resuspended in a high K medium (1 mg protein/ml) containing 135 mM KCl 1 0 mM MgCl 1 2 mM KH PO 0 1 mM phenylmethylsulphonyl fluoride 0 2 mg/ml soybean trypsin inhibitor and 10 mM HEPES (pH 7 4) were permeabilized with saponin (30 µg/ml for 10 min at 25°C) as previously described [39 40]

Ca uptake and release experiments in permeabilized acinar cells Permeabilized pancreatic acinar cells were washed twice and resuspended in a Ca uptake medium (4 mg protein/ml) containing 120 mM KCl 1 0 mM MgCl 1 2 mM KH PO 5 mM pyruvate 5 mM succinate 0 5 mM EGTA 0 5 mM nitrilotriacetic acid 0 5 mM HEEDTA 0 2 mg/ml soybean trypsin inhibitor and 20 mM HEPES adjusted with KOH to pH 7 1 Permeabilized cells were kept on ice until use Ca uptake was started by adding 10 µl of permeabilized cells to 90 µl of Ca^{2} uptake medium which contained in addition 10 mM creatine phosphate 10 U/ml creatine kinase 0 mM or 1 0 mM NaATP 3% (w/v) polyethylene glycol (mol wt 4000) and 1 9×10⁵ Bq/ml ^{45}Ca The free Mg (0 89 mM) and free Ca (0 19 µM) concentrations were adjusted as described by Schoenmakers et al [32] The incubations performed at 37°C were stopped at the indicated times by adding 1 0 ml of ice cold stop solution containing 150 mM KCl 5 0 mM MgCl 1 0 mM EGTA and 20 mM HEPES/KOH (pH 7 1) The suspension was rapidly filtered (Schleicher and Schull GF92) The filters were washed twice with 1 0 ml of ice cold stop solution dissolved in scintillation fluid and counted for radioactivity Total Ca was calculated and expressed as nmol/mg protein Actively stored Ca is defined as the difference in total Ca retained on the filter after incubation in the presence and absence of ATP Protein was determined with a commercial Coomassie blue kit (Bio Rad Richmond Calif USA) after treatment of the cells with 0 1% Triton X 100

Results

Selective depletion of the Ins(1 4 5)P_3 sensitive Ca^{2+} store by U73122 in permeabilized pancreatic acinar cells

Isolated pancreatic acinar cells permeabilized by saponin treatment rapidly accumulate Ca^{2+} when incubated in

the continuous presence of ATP [39, 40]. Maximal loading of the internal stores occurs within 10 min and remains unchanged thereafter. Complete inhibition of the Ca^{2+} pump by thapsigargin results in a gradual loss of Ca^{2+} with a $t_{1/2}$ of approximately 6 min[1], indicating that the plateau reached after 10 min of loading is the result of a steady-state between slow Ca^{2+} efflux and ATP-driven Ca^{2+} uptake. Since U73122 was found to increase $[Ca^{2+}]_{i,av}$ in a thapsigargin-like manner (Fig. 2), the effects of this compound on Ca^{2+} uptake and Ca^{2+} release were investigated in the permeabilized acinar cell preparation. When loaded in the continuous presence of U73122, a dose dependent decrease of the steady-state level was observed (Fig. 1 a). Subsequent stimulation with a maximal concentration of 10 μM Ins(1,4,5)P_3 revealed that U73122 preferentially inhibited ATP-dependent Ca^{2+} uptake in the Ins(1,4,5)P_3-sensitive store, with an IC_{50} of approximately 4 μM. By contrast, thapsigargin was previously shown to equally inhibit ATP-dependent Ca^{2+} uptake in both stores [40]. Addition of U73122 (6 μM) to permeabilized acinar cells loaded with Ca^{2+} to steady-state resulted in a rapid loss of accumulated Ca^{2+} (Fig. 1b). During the first 2 min following the addition of U73122 the release rate was significantly higher than that obtained with 1 μM thapsigargin. Interestingly, the effects of U73122 and thapsigargin were partly additive. Since thapsigargin completely inhibits ATP-dependent Ca^{2+} uptake when added at a concentration of 1 μM, these results suggest that U73122 does not act at the level of the Ca^{2+} pump alone. In addition, the Ins(1,4,5)P_3 receptor antagonist heparin [11] did not affect U73122-stimulated Ca^{2+} release, indicating that this receptor is not involved in the mechanism of action of U73122. In the presence of heparin (200 U/ml), 6 μM U73122 released 37.6% (SEM ± 6.8%, $n = 5$) of actively stored Ca^{2+} in 4.5 min, whereas in the absence of heparin the drug released 42.3% (SEM ± 5.1%, $n = 5$). Likewise, the stimulatory effect of U73122 remained unimpaired by either 20 mM caffeine or 10−50 μM ryanodine (data not shown).

Effects of U73122 on resting levels, and JMV-180-evoked increases, of the average $[Ca^{2+}]_i$ in a suspension of pancreatic acinar cells

The putative inhibitor of phospholipase C, U73122 both dose dependently increased $[Ca^{2+}]_{i,av}$ and inhibited the stimulatory effect of JMV-180 added thereupon (Fig. 2 c−f). The effect of U73122 alone was clearly transient, in that a relatively fast increase was followed by a markedly slower decrease to resting (d) or slightly elevated (e and f) values. The minimal effective concentration of U73122 to increase $[Ca^{2+}]_{i,av}$ was 3 μM (Fig. 2 d). The magnitude of the peak increase in $[Ca^{2+}]_{i,av}$ obtained with CCK_8 and JMV-180 was dose dependent (Fig. 3 a, b). The maximal effect obtained with JMV-180 was approximately 50% of that obtained with CCK_8 (see also [30]). The half-maximal stimulatory concentrations of JMV-180 and CCK_8 were 10 nM and

[1] F.H.M.M. Van de Put, J.J.H.H.M. de Pont and P.H.G.M. Willems, unpublished observations.

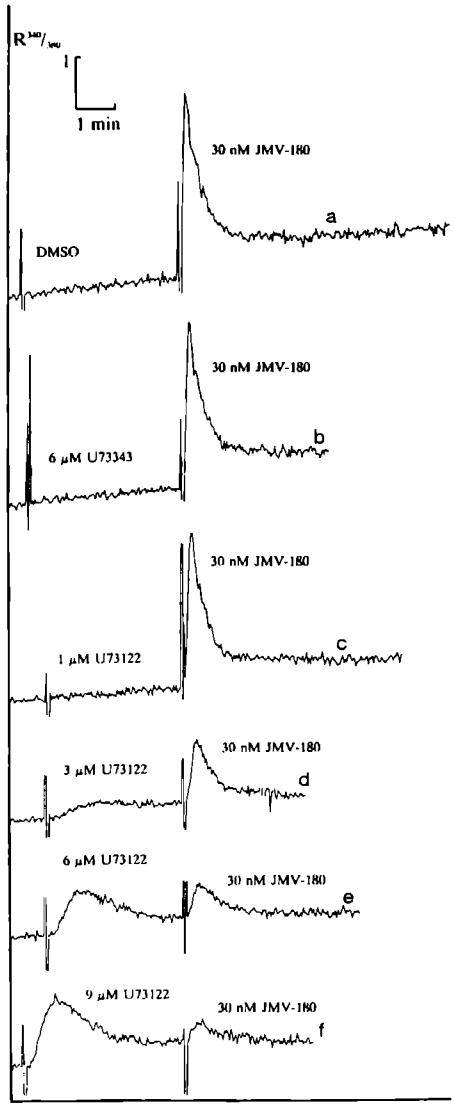

Fig. 2 a–f. Inhibition of the JMV-180-evoked increase in the average intracellular Ca^{2+} concentration ($[Ca^{2+}]_{i,av}$) by U73122. Rabbit pancreatic acinar cells loaded with Fura-2 and resuspended in HEPES/TRIS medium (pH 7.4) containing 0.1% bovine serum albumin, were transferred to a cuvette placed in a spectrofluorophotometer and fluorescence was measured at 37°C with continuous stirring. Dimethyl sulphoxide (DMSO), U73343, U73122 and JMV-180 were added at times indicated and at the final concentrations given in the figure. JMV-180 was added in addition to the compounds already present. The fluorescence emission ratio at 490 nm was monitored as a measure of $[Ca^{2+}]_{i,av}$ after excitation at 340 and 380 nm

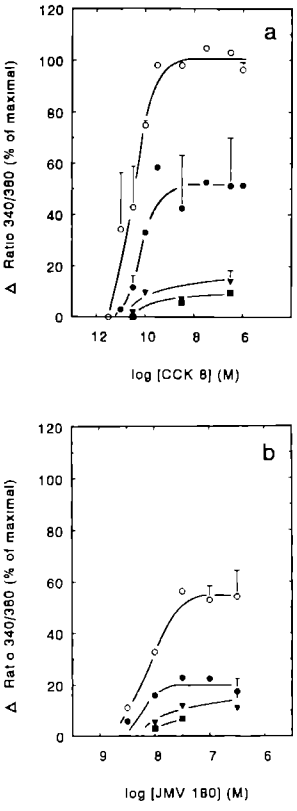

Fig 3 a, b Effect of U73122 on the dose/response curves for the JMV 180 and cholecystokinin octapeptide (CCK_8) evoked increases in peak fluorescence emission ratio 340/380 nm Fluorescence measurements were performed as described in the caption of Fig 1 At 3 min following the addition of DMSO (*open circles*) or U73122 (3 μM *closed circles* 6 μM *closed triangles* 9 μM *closed squares*) the suspensions were stimulated with either CCK_8 (**a**) or JMV 180 (**b**) at the indicated concentrations In each experiment the maximal peak increase in fluorescence emission ratio 340/380 nm obtained with 1 nM CCK_8 is set at 100% to which all other values are related Where indicated the values presented are the mean ± SEM of 3 independent determinations In most other cases the values presented are the mean of 2 independent measurements The values presented were obtained with 6 different cell preparations

30 pM respectively U73122 dose dependently reduced the maximal effect of both JMV 180 and CCK_8 without significantly affecting the respective EC_{50} values Maximal inhibition by approximately 90% was reached with 6 μM U73122 The biologically inactive analogue of U73122 U73343 (6 μM), neither changed the resting $[Ca^{2+}]_{av}$ nor affected subsequent stimulation with 30 nM JMV 180 (Fig 2 b)

Differential depletion of agonist sensitive and insensitive Ca stores by U73122 and thapsigargin as measured in a suspension of pancreatic acinar cells

Experiments performed in the presence of 0 5 mM EGTA and absence of added Ca demonstrated that U73122 mobilized Ca from intracellular stores (Fig 4 a–c) At a concentration of 10 μM U73122 evoked a relatively fast increase in [Ca^{2}] followed by a somewhat slower decrease to the basal level This decrease, however was markedly faster than observed in the presence of external Ca (for comparison see Fig 2 e f) This decrease (Fig 6 a) was also markedly faster than the decrease obtained with 1 μM thapsigargin (Fig 6 b c)

Stimulation with a maximally effective concentration of either 1 μM CCK_8 (Fig 4 a) or 10 μM JMV 180 (Fig 4 b) at 2 5 min following the addition of either U73122 or thapsigargin revealed that 10 μM U73122 almost completely blocked the response to both secretagogues whereas in suspensions prestimulated with 1 μM thapsigargin both CCK_8 (Fig 4 d) and JMV 180 (Fig 4 e) were still able to evoke a marked though reduced transient increase in [Ca]$_a$ Since the thapsigargin and U73122 evoked [Ca]$_a$ transients were equal sized these observations suggest that U73122 either inhibits agonist evoked Ins(1 4 5)*P* formation or selectively depletes the agonist sensitive store

Prestimulation with 1 μM CCK_8 led to a marked decrease in size of the [Ca] transient evoked by subsequent addition of 10 μM U73122 (Fig 5 a) However when the cell suspension was subsequently stimulated with 1 μM thapsigargin the magnitude of the response was similar to that obtained with suspensions of cells prestimulated with 10 μM U73122 alone (for comparison see Figs 4 c and 6 a) On the other hand in a suspension of cells prestimulated with 10 μM JMV 180 which evoked a considerably smaller response than 1 μM CCK_8 subsequent addition of 10 μM U73122 led to a markedly more pronounced increase in [Ca]$_a$ than observed after prestimulation with 1 μM CCK_8 (Fig 5 b) Again subsequent stimulation with 1 μM thapsigargin resulted in a [Ca]$_a$ transient the magnitude of which was similar to that obtained after a single addition of 10 μM U73122 This inverse relationship in size between the agonist and U73122 evoked [Ca]$_a$ transient clearly demonstrates that U73122 selectively mobilizes Ca from the agonist sensitive store leaving unaffected the agonist insensitive store released by the subsequent addition of thapsigargin

Thus far the reason for the reduced efficacy of JMV 180 (see also [30]) is unclear However the above observation that U73122 evokes a substantially larger increase in [Ca] in cells prestimulated with 10 μM JMV 180 than in cells prestimulated with 1 μM CCK_8 suggests that JMV 180 can only partly release the agonist sensitive Ca store This explanation is supported by the observation that carbachol can still evoke a substantial increase in [Ca] in a suspension of acinar cells pre

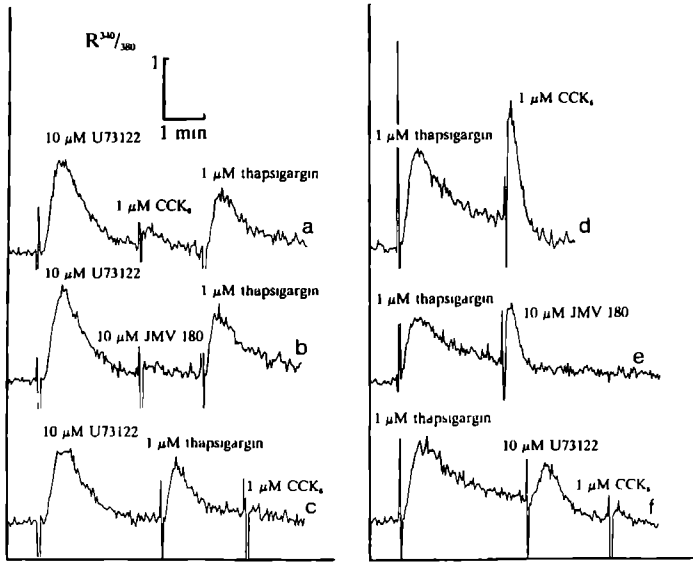

Fig. 4 a–f. Effect of U73122 and thapsigargin pretreatment on the relative sizes of the agonist-sensitive and -insensitive internal Ca^{2+} stores Fura 2 loaded pancreatic acinar cells, resuspended in HEPES/TRIS medium (pH 7.4) to which no Ca^{2+} was added, were transferred to the thermostatic cuvette and the external Ca^{2+} concentration was further reduced by the addition of 0.5 mM EGTA. U73122, thapsigargin, JMV-180 and CCK_8 were added consecutively. The compounds were added at the indicated concentrations and at the indicated times.

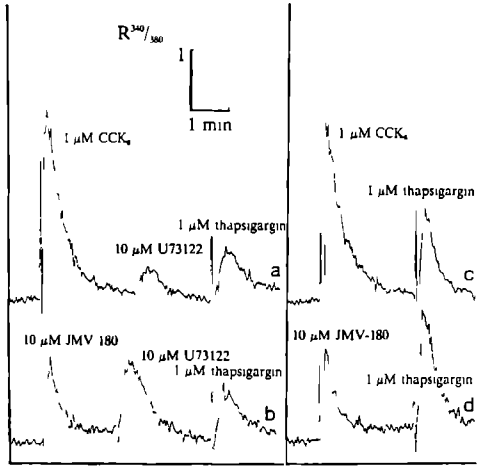

Fig. 5 a–d. Selective depletion of the agonist-sensitive internal Ca^{2+} store by U73122. Fluorescence measurements were performed in nominally Ca^{2+}-free medium as described in the caption of Fig. 3. CCK_8 (1 μM), JMV-180 (10 μM), U73122 (10 μM) and thapsigargin (1 μM) were added consecutively at the indicated times.

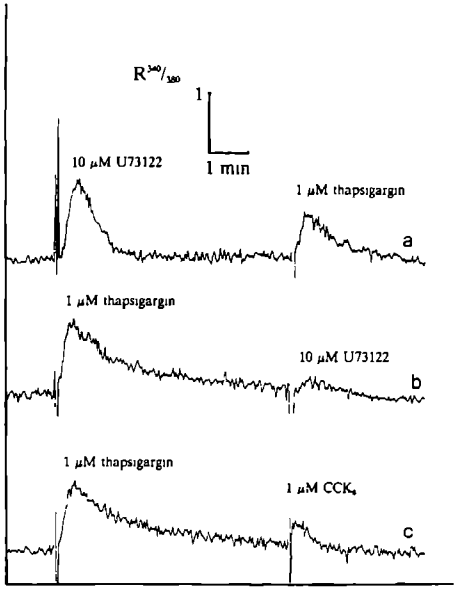

Fig. 6 a–c. Effects of prolonged incubation with U73122 and thapsigargin on the relative sizes of the agonist-sensitive and -insensitive internal Ca^{2+} stores. Fluorescence measurements were performed in nominally Ca^{2+} free medium as described in the caption of Fig. 3. U73122 (10 μM), thapsigargin (1 μM) and CCK_8 (1 μM) were added consecutively at the indicated times.

stimulated with a maximally effective concentration of JMV-180 [45].

Interestingly, when 1 μM thapsigargin was added to cells prestimulated with either 1 μM CCK_8 or 10 μM

Table 1. Dose/response curve for the induction of sustained Ca^{2} oscillations by U73122

Conditions	Concentration (μM)	Oscillating cells (% of total)	
U73122	0	0	(n − 1)
	3	32	(SD ± 7 n − 3)
	6	54	(SD ± 8, n = 3)
U73343	6	0	(n = 1)

Rabbit pancreatic acinar cells loaded with Fura 2 and resuspended in HEPES/TRIS medium (pH 7 4) containing 0 1% bovine serum albumin were stimulated with either U73122 or U73343 at the indicated concentrations and transferred to the thermostatic (32°C) incubation chamber The cells were allowed to equilibrate for 6–10 min before fluorescence measurement was started The measuring time was 10 min After background correction and calculation of the fluorescence ratio, the changes in fluorescence emission ratio in a randomly selected group of 37 (SD + 11 n = 11) cells were analysed The data presented were obtained with three different cell preparations

JMV-180 the shape of the $[Ca^{2+}]_{av}$ transient differed essentially from that obtained with thapsigargin alone, in that both the rising and falling phases were markedly accelerated (Fig 5 c, d, for comparison, see Figs 4 d–f and 6 b, c) A possible explanation for this phenomenon is that in the continuous presence of agonist the agonist sensitive Ca^{2+} stores are still partly filled due to compensatory Ca^{2} pumping Addition of thapsigargin then results in the immediate cessation of compensatory Ca^{2+} pumping and the rapid release of Ca^{2+} through the open $Ins(1,4,5)P_3$-operated Ca^{2+} release channels

Increasing the preincubation time with 1 μM thapsigargin to 6 min resulted in a significant reduction of the response to both 10 μM U73122 and 1 0 μM CCK_8 (Fig 6 b, c, for comparison, see Fig 4 f, d) This is consistent with the previous observation that thapsigargin depletes both the agonist-sensitive and -insensitive Ca^{2+} stores [40] On the other hand, prolonged stimulation with 10 μM U73122 did not lead to a similar reduction in the magnitude of the thapsigargin-evoked increase in $[Ca^{2}]_{av}$ (Fig 6 a, for comparison, see Fig 4 a–c) These observations suggest that U73122 specifically affects the hormone-sensitive and not the hormone-insensitive part of the thapsigargin-releasable pool

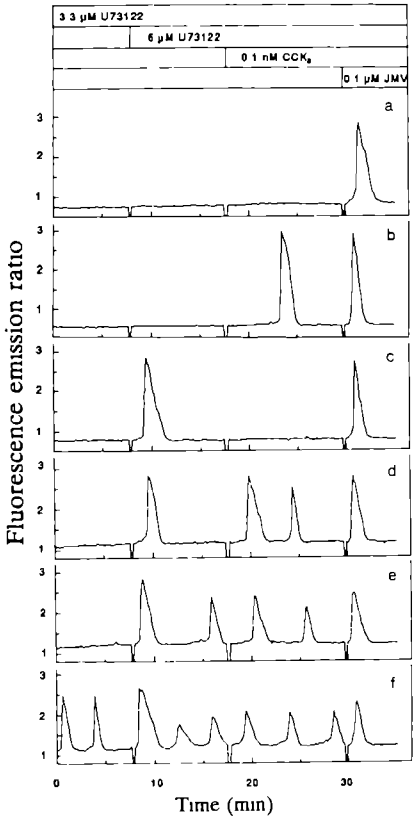

Fig. 7 a–f. Induction of sustained Ca^{2} oscillations by U73122 Fura 2 loaded pancreatic acinar cells stimulated with 3 μM U73122 were transferred to the thermostatic (32°C) incubation chamber and allowed to equilibrate for 10 min Changes in $[Ca^{2}]$ were monitored by digital imaging microscopy of Fura 2 fluorescence from individual acinar cells The recordings shown are from 6 individual acinar cells monitored simultaneously U73122 to a final concentration of 6 μM CCK_8 and JMV 180 were added by gentle dropping onto the incubation medium at the times indicated

Induction of cytoplasmic Ca^{2+} oscillations by U73122 in single pancreatic acinar cells

Digital imaging microscopy of Fura-2-loaded pancreatic acinar cells was used to study the effects of U73122 on Ca^{2+} homeostasis in both stimulated and unstimulated single pancreatic acinar cells When added to unstimulated acinar cells, U73122 alone evoked oscillatory changes in $[Ca^{2+}]_i$ (Figs 7 e, f and 8) U73122 evoked Ca^{2+} oscillations could be monitored for at least 40 min, indicating their sustained nature (data not shown) The number of acinar cells displaying these distinct Ca^{2+} oscillations depended on the concentration of the drug used (Table 1) In the presence of 6 μM U73122 54% (SEM ± 8%, n = 3) of the acinar cells displayed oscillatory changes in $[Ca^{2+}]_i$ This heterogeneity among acinar cells is further illustrated in detail in Fig 7 The recordings a and b are from cells which did not display U73122-induced changes in $[Ca^{2+}]_i$ during the period of monitoring, whereas the cells c–e showed no oscillations in response to 3 3 μM U73122, but were stimulated upon increasing the final concentration of the drug to 6 0 μM U73343 was ineffective in inducing Ca^{2} oscillations (Table 1)

The effectiveness of U73122 in inhibiting receptor mediated Ca^{2+} mobilization was tested by subsequent addition of 0 1 nM CCK_8 at 20 min and 0 1 μM JMV-

Table 2 Inhibition of JMV 180 and carbachol evoked Ca mobilization in individual pancreatic acinar cells by U73122

U73122 concentration (μM) added at 0 min	Responding cells (% of total)		
	U73122 alone	0 1 μM JMV 180 added at 16 min	3 μM carbachol added at 21 min
0	0	81/73	100/97
3	33	58	75
6	50	64	59

Rabbit pancreatic acinar cells loaded with Fura 2 and resuspended in HEPES/TRIS medium (pH 7 4) containing 0 1% bovine serum albumin were stimulated with U73122 at the indicated concentrations and then transferred to the thermostatic (32°C) incubation chamber The cells were allowed to equilibrate for 6 min before fluorescence measurement was started Measuring time was 20 min At 16 min following the addition of U73122 the cells were stimulated with JMV 180 at a final concentration of 0 1 μM Carbachol was added at a final concentration of 3 μM at 21 min following the addition of U73122 Both agonists were added from a 7 5 times concentrated stock solution by gentle dropping stock onto the incubation medium After background correction and calculation of the fluorescence ratio the changes in fluorescence emission ratio in a randomly selected group of 27 (SD + 5 $n = 4$) cells were analysed The control experiment was performed in duplicate The data presented were obtained with the same cell preparation

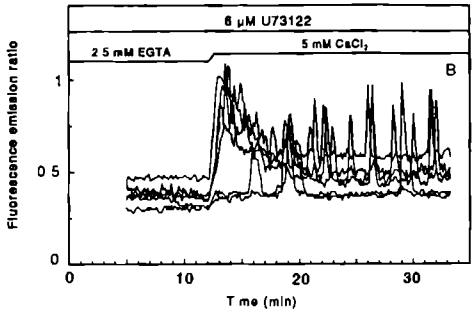

Fig 8 Cessation of U73122 evoked Ca oscillations by external Ca^{2} removal Fura 2 loaded pancreatic acinar cells resuspended in medium to which no Ca was added and which contained in addition 2 5 mM EGTA were stimulated with 6 μM U73122 and transferred to the thermostatic (32°C) incubation chamber Monitoring was started at 5 min CaCl (5 mM) was added in addition to EGTA at the indicated time The recordings shown are from 6 individual acinar cells monitored simultaneously

180 at 30 min (Fig 7) In cells not responding to U73122 both CCK_8 (Fig 7 b) and JMV 180 (Fig 7 a) could evoke a transient increase in [Ca] suggesting that the process of receptor mediated Ca^{+} mobilization was not inhibited sufficiently in these particular cells In cells displaying U73122 evoked Ca^{2} oscillations it was difficult to unambiguously conclude that the pathway leading to receptor evoked Ca^{+} mobilization was indeed interrupted Some cells displaying U73122 induced Ca oscillations of a low frequency apparently responded to CCK_8 (Fig 7 d) and/or JMV 180 (Fig 7 c d) In cells showing U73122 induced Ca oscillations of higher frequencies it was impossible to demonstrate stimulatory effects of CCK_8 (Fig 7 e f) JMV 180 (Fig 7 e f) and/or carbachol (not shown)

Table 2 shows that addition of 0 1 μM JMV 180 to acinar cells preincubated in the presence of U73122 for 10 min further increased the number of cells displaying periodic changes in $[Ca^{2+}]$ However the percentage oscillating cells in the combined presence of U73122 and JMV 180 was less than in the presence of JMV 180 alone Subsequently the cells were stimulated with 3 μM carbachol In the control experiment 98% of the cells responded in the combined presence of JMV 180 and carbachol whereas in the continuous presence of U73122 the number of cells responding in the combined presence of JMV 180 and carbachol was reduced These data indicate that U73122 inhibits agonist-evoked Ca mobilization in part of the acinar cells, leaving other cells unimpaired

By contrast thapsigargin only extremely rarely evoked sustained oscillatory changes in [Ca] (data not shown) In addition the drug completely inhibited receptor mediated Ca^{2} mobilization at concentrations at or beyond 30 nM indicating that the agonist sensitive Ca^{2+} stores were completely depleted by the action of thapsigargin

Dependence of U73122 evoked cytoplasmic Ca^{2+} oscillations on external Ca^{+}

Acinar cells resuspended in a HEPES/TRIS medium to which no Ca^{2} was added and which contained in addition 2 5 mM EGTA did not display Ca^{2+} oscillations at 5 min following stimulation with 6 μM U73122 (Fig 8) Readdition of Ca^{2} to the medium resulted in a rapid increase of the basal $[Ca^{+}]$ and the recurrence of the U73122 evoked Ca oscillations The latter observation clearly demonstrates that external Ca^{+} plays a crucial role in the induction of U73122 evoked Ca^{2+} oscillations

Discussion

The experiments described here aimed to investigate the effects of the putative phospholipase C inhibitor U73122 on the process of receptor mediated Ca^{2} mobilization in the rabbit pancreatic acinar cell The main finding of the present study is that U73122 selectively reduced the size of the agonist sensitive part of the intracellular Ca^{2+} store This action was paralleled by the induction of sustained oscillatory changes in $[Ca^{2+}]$ Both effects of U73122 were dose dependent and not observed with the biologically inactive analogue U73343

In a suspension of acinar cells U73122 dose dependently reduced the size of both the CCK_8 and the JMV

180-evoked $[Ca^{2+}]_{av}$ transient However the observation that U73122 alone could induce a transient increase in $[Ca^{2+}]_{av}$ the size of which was inversely related to that evoked by agonists added previously, suggests that the inhibitory effect of U73122 was caused by depletion of the agonist-sensitive Ca^{2+} store rather than by inhibition of phospholipase C activity

Video-imaging microscopy of Fura-2-loaded acinar cells revealed that U73122 could evoke oscillatory changes in $[Ca^{2+}]_i$ However, individual acinar cells displayed large differences in sensitivity to U73122 as was reflected by the dose dependent recruitment of cells displaying U73122-evoked Ca^{2+} oscillations The occurrence of U73122-evoked Ca^{2+} oscillations hampered the assessment of its effect on agonist-induced Ca^{2+} mobilization

U73122 has been reported to inhibit ongoing Ca^{2+} oscillations in pancreatic acinar cells stimulated by CCK_8 but not by JMV-180, without affecting $[Ca^{2+}]_i$ by itself [46] Thus far, the reason for the discrepancy between the observations presented in this study and those reported in literature is unclear However, the possibility of species differences, rat [46, 48] versus rabbit, playing a role can not be ruled out Another difference between both studies is the use of a preparation consisting of single acinar cells and relatively small acini [44] in the present study, rather than isolated acini [46, 48]

The most intriguing observation of the present study is the ability of U73122 to evoke sustained Ca^{2+} oscillations by itself Although the initial U73122-evoked increase in $[Ca^{2+}]_{av}$ was independent of extracellular Ca^{2+}, subsequent oscillations were abolished in the absence of external Ca^{2+} We have previously shown that permeabilized acinar cells accumulate Ca^{2+} in ATP-dependent Ca^{2+} stores to a steady-state level, which is reached at 10 min following the addition of ATP [39, 40] About 60% of this actively stored Ca^{2+} can be released by Ins(1,4,5)P_3, whereas the remainder resides in a store insensitive to this messenger The data presented in this study show that U73122, in contrast to thapsigargin [40], which completely inhibited ATP-dependent Ca^{2+} accumulation [40], can cause the selective depletion of the Ins(1,4,5)P_3-sensitive store The effect of U73122 was not affected by heparin, indicating that Ins(1,4,5)P_3 was not involved in U73122-stimulated Ca^{2+} release

Based on the above observations, we propose the following explanation for the ability of U73122 to evoke sustained external Ca^{2+}-dependent Ca^{2+} oscillations in a non-excitable cell type such as the pancreatic acinar cell U73122 evoked depletion of the Ins(1,4,5)P_3-sensitive store results in an increase of the plasma membrane permeability for Ca^{2+} as originally postulated by Putney and co workers [26, 27, 34] Ca^{2+} entering the cell via the plasma membrane will be accumulated in Ca^{2+} stores insensitive to Ins(1,4,5)P_3, but sensitive to thapsigargin Once these stores are filled, cytosolic Ca^{2+} will increase and trigger the mechanism of CICR Ca^{2+} will be released rapidly from the Ins(1,4,5)P_3-insensitive store leading to a dramatic increase in $[Ca^{2+}]_i$ Part of the Ca^{2+} will be extruded and part will be reaccumulated in the Ins(1,4,5)P_3-insensitive store Now, a new cycle of events leading to the massive release of Ca^{2+} can start In the absence of U73122, the Ins(1,4,5)P_3-sensitive stores will also accumulate Ca^{2+} and the plasma membrane permeability for Ca^{2+} will decrease thus preventing the occurrence of the process of CICR The observation that thapsigargin was still able to evoke a transient rise in $[Ca^{2+}]_i$ in a suspension of cells prestimulated with 10 μM U73122 in the absence of external Ca^{2+} (Fig 6 a), supports our idea that in the presence of U73122 the agonist-insensitive store will only be depleted upon Ca^{2+} entering the cell

According to the above hypothesis, the observation that thapsigargin was unable to induce sustained Ca oscillations in pancreatic acinar cells can be explained by the fact that it inhibits Ca^{2+} accumulation in both the Ins(1,4,5)P_3-sensitive and Ins(1,4,5)P_3-insensitive pools thus preventing loading of the store involved in the process of CICR Interestingly, in parotid acinar cells, in which thapsigargin was found to be able to induce Ca^{2+} oscillations, the drug was shown to be unable to inhibit the uptake of Ca^{2+} in the Ins(1,4,5)P_3-insensitive pool [8–10] The data obtained with U73122 provide additional evidence for the existence of CICR in pancreatic acinar cells, whereas in addition they provide evidence that the depleted Ins(1,4,5)P_3-sensitive Ca^{2+} store can signal to the plasma membrane in the absence of hormone, or without prestimulation by a hormone Moreover, they demonstrate that Ca^{2+} oscillations can occur under conditions when phospholipase C is inhibited

The present study suggests that the minimum requirements for the occurrence of cytosolic Ca^{2+} oscillations are (1) an empty agonist sensitive store, stimulating the influx of external Ca^{2+}, (2) a functional agonist-insensitive store, accumulating Ca^{2+} entering the cytosol, and (3) the presence of a Ca^{2+}-sensitive Ca^{2+} release channel, allowing Ca^{2+} to be released from the Ins(1,4,5)P_3-insensitive store as $[Ca^{2+}]_i$ increases According to the above model, the role of Ins(1,4,5)P_3 is to deplete the agonist-sensitive intracellular Ca^{2+} store in order to turn on the oscillatory mechanism This idea is supported by the observation that Ca^{2+} oscillations are maintained in the continuous presence of elevated cellular Ins(1,4,5)P_3 levels [41] In this view, fluctuations in the cellular Ins(1,4,5)P_3 level [4] and/or sensitivity of the Ins(1,4,5)P_3-operated Ca^{2+} release channel to activation by Ins(1,4,5)P_3 [6, 19, 49] lead to a continuous turning on and off of the oscillatory mechanism

Acknowledgement The research of Dr P H G M Willems has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences

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Chapter 6

Heterogeneity between intracellular Ca^{2+} stores as the underlying principle of quantal Ca^{2+} release by inositol 1,4,5-trisphosphate in permeabilized pancreatic acinar cells

In: Journal of Biological Chemistry, 269, 12438 -12443 (1994)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol 269 No 17 Issue of April 29 pp 12438–12443 1994
Printed in U S A

Heterogeneity between Intracellular Ca^{2+} Stores as the Underlying Principle of Quantal Ca^{2+} Release by Inositol 1,4,5-Trisphosphate in Permeabilized Pancreatic Acinar Cells*

(Received for publication, November 29, 1993, and in revised form, February 2, 1994)

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Permeabilized rabbit pancreatic acinar cells were used to study the effects of Ca^{2+} pump inhibition and Ca^{2+} store depletion on the sensitivity of internal Ca^{2+} stores to emptying by inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3). Complete inhibition of pump activity by thapsigargin resulted in the monoexponential loss of 92% of the actively stored Ca^{2+} with a half-time of 6.2 min. Under these conditions, Ca^{2+} release evoked by a submaximal concentration of Ins-1,4,5-P_3 did not cease after 1.5 min, as was observed in the absence of thapsigargin, but continued for at least 5 min. This observation suggests that under normal conditions of Ca^{2+} pumping, a substantial part of the internal Ca^{2+} stores is not depleted by the action of Ins-1,4,5-P_3 due to compensatory Ca^{2+} uptake. Evidence in support of the idea of compensatory Ca^{2+} pumping was obtained in exchange experiments performed in the absence of thapsigargin. The slow kinetics of sustained Ca^{2+} release in the absence of Ca^{2+} pump activity suggests that Ca^{2+} is released from stores containing either relatively few or less sensitive Ins-1,4,5-P_3-operated Ca^{2+} release channels. Gradual emptying of the internal Ca^{2+} stores by thapsigargin did not affect the potency with which Ins-1,4,5-P_3 released Ca^{2+}, indicating that the intravesicular Ca^{2+} content does not control the sensitivity of the Ins-1,4,5-P_3-operated Ca^{2+} channel to activation by Ins-1,4,5-P_3. This was confirmed using ruthenium red, which preferentially depleted the Ins-1,4,5-P_3-releasable store without affecting the EC_{50} for Ins-1,4,5-P_3-stimulated Ca^{2+} release. The data presented indicate that the quantal type of Ca^{2+} release observed with Ins-1,4,5-P_3 requires compensatory Ca^{2+} pumping. Moreover, they support the idea that internal Ca^{2+} stores display differential sensitivities toward Ins-1,4,5-P_3 rather than responding uniformly to this internal Ca^{2+}-mobilizing messenger.

Permeabilized cells have been used extensively to study the characteristics of Ins-1,4,5-P_3 [1]-stimulated Ca^{2+} release from intracellular stores In the presence of ATP, these stores rapidly accumulate Ca^{2+} until a steady state is reached between passive Ca^{2+} efflux and active Ca^{2+} uptake As a consequence, the steady-state level will immediately change upon altering one or both parameters Complete depletion of the store requires either complete inhibition of the Ca^{2+} pump or the introduction of a Ca^{2+} leak, which cannot be compensated for by the Ca^{2+} pump

One of the intriguing features of the process of Ins-1,4,5-P_3-stimulated Ca^{2+} release is that it is quantal by nature (1, 2), *i e* stimulation with a submaximal concentration of Ins-1,4,5-P_3 leads to the instantaneous release of only a fraction of the Ins-1,4,5-P_3-sensitive Ca^{2+} pool Basically, there are two explanations for this phenomenon First, submaximal concentrations of Ins-1,4,5-P_3 may completely release a fraction of the Ca^{2+} stores, and the unaffected stores are depleted only upon further increasing the Ins-1,4,5-P_3 concentration (1, 3–5) Second, submaximal concentrations of Ins-1,4,5-P_3 may partially release all stores to the same extent, and the remainder of the stored Ca^{2+} is not released before further increasing the Ins-1,4,5-P_3 concentration (6)

Recently, the latter explanation has attracted much attention, and a model has been put forward in which the intravesicular Ca^{2+} content controls the sensitivity of the Ins-1,4,5-P_3-operated Ca^{2+} release channel to activation by Ins-1,4,5-P_3 (6) According to this model, a decrease in the Ca^{2+} content within the stores would lead to a decrease in affinity of the Ins-1,4,5-P_3 receptor for its ligand Evidence in support of this model comes from experiments in which intracellular Ca^{2+} stores in permeabilized hepatocytes were overloaded with Ca^{2+}, leading to an increase in the sensitivity of the Ins-1,4,5-P_3-operated Ca^{2+} release channel to activation by Ins-1,4,5-P_3 (7) Additional evidence comes from experiments in which intracellular Ca^{2+} stores were depleted by the action of either Ca^{2+} ionophores, such as ionomycin (8), or inhibitors of Ca^{2+}-ATPase activity, such as thapsigargin (9, 10) These experiments showed an increase in the half-maximal stimulatory Ins-1,4,5-P_3 concentration at lower luminal Ca^{2+} contents However, other studies did not lead to this conclusion (11–13)

In this study, isolated pancreatic acinar cells permeabilized by saponin treatment were used to investigate a possible role of intravesicular Ca^{2+} in regulating the sensitivity of the Ins-1,4,5-P_3-operated Ca^{2+} channel to activation by Ins-1,4,5-P_3 To exclude possible stimulatory or inhibitory effects of Ca^{2+} at the cytosolic side of the receptor (14–17), experiments were performed at a fixed ambient free Ca^{2+} concentration of 190 nM To decrease the intravesicular Ca^{2+} content, permeabilized cells were treated with either thapsigargin, which effectively blocks intracellular Ca^{2+} pump activity in a noncompetitive manner (18), or ruthenium red, which predominantly reduces the size of the Ins-1,4,5-P_3-sensitive Ca^{2+} pool (19)

The data presented do not support the idea that intravesicular Ca^{2+} plays a regulatory role in the process of Ins-1,4,5-P_3-induced Ca^{2+} mobilization By contrast, they demonstrate that the quantal nature of Ins-1,4,5-P_3-stimulated Ca^{2+} release is most likely explained by store heterogeneity in that a submaximal concentration of Ins-1,4,5-P_3 rapidly depletes those stores in which it evokes a relatively high Ca^{2+} leak, which cannot be

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[1] The abbreviations used are Ins 1,4 5-P_3, inositol 1 4 5-trisphosphate HEEDTA, *N*-hydroxyethylethylenediaminetriacetic acid

compensated for by active Ca^{2+} uptake, whereas in other stores in which it evokes a smaller Ca^{2+} leak, enhanced Ca^{2+} release can be completely or partially compensated for by increased pumping activity

EXPERIMENTAL PROCEDURES

Pancreatic Acinar Cells—Rabbit pancreatic acinar cells were prepared according to the method of Amsterdam and Jamieson (20) as previously described (21, 22)

Permeabilization of Acinar Cells—Isolated pancreatic acinar cells were permeabilized with saponin as previously described (19, 21, 22) Briefly, acinar cells were washed twice, resuspended in high K^+ medium (1 mg of protein/ml) containing 135 mM KCl 1 0 mM $MgCl_2$, 1 2 mM KH_2PO_4, 0 1 mM phenylmethanesulfonyl fluoride, 0 2 mg/ml soybean trypsin inhibitor, and 10 mM Hepes (pH 7 4), and permeabilized with saponin (30 μg/ml, 10 min, 25 °C)

Ca^{2+} Uptake and Release Experiments—Permeabilized acinar cells were washed twice, resuspended in Ca^{2+} uptake medium (4 mg of protein/ml) containing 120 mM KCl, 1 0 mM $MgCl_2$, 1 2 mM KH_2PO_4, 5 mM pyruvate, 5 mM succinate, 0 5 mM EGTA, 0 5 mM nitrilotriacetic acid, 0 5 mM HEEDTA, 0 2 mg/ml soybean trypsin inhibitor, and 20 mM Hepes, and adjusted to pH 7 1 with KOH The permeabilized cells were kept at 0 °C Ca^{2+} uptake was started by adding 10 μl of permeabilized cells to 90 μl of warmed (37 °C) Ca^{2+} uptake medium that contained, in addition, 10 mM phosphocreatine, 10 units/ml creatine kinase, 0 or 1 mM NaATP, 3% (w/v) polyethylene glycol (M_r 4000), and 5 μCi/ml $^{45}Ca^{2+}$ The free Mg^{2+} (0 89 mM) and Ca^{2+} (0 19 μM) concentrations were adjusted as described by Schoenmakers *et al* (23) Incubation was stopped by adding 1 0 ml of ice-cold stop solution containing 150 mM KCl, 5 0 mM $MgCl_2$, 1 0 mM EGTA, and 20 mM Hepes/KOH (pH 7 1), and the suspension was rapidly filtered (Schleicher & Schull, GF92) The filters were washed with 2 × 1 0 ml of ice cold stop solution, dissolved in scintillation fluid, and counted for radioactivity Total Ca^{2+} was calculated and is expressed as nanomoles/milligram of protein Actively stored Ca^{2+} is defined as the difference in total Ca^{2+} retained on the filter after incubation in the presence and absence of ATP

Protein was determined with a commercial Coomassie Blue kit (Bio Rad) after treatment of the cells with 0 1% Triton X-100 γ-Globulin (Bio-Rad) was used as a standard

Analysis of Data—The EC_{50} was determined by fitting the concentration-response relationship to a logistic equation using the nonlinear regression computer program InPlot (GraphPAD Software for Science, San Diego, CA)

The kinetics with which the Ca^{2+} content of the energy-dependent Ca^{2+} store decayed following the addition of thapsigargin could be fitted to the following equation $Ca^{2+}(t) = Ca^{2+}(t = 0) \exp(-\lambda t) + Ca^{2+}(B)$, where $Ca^{2+}(t)$ is the Ca^{2+} content in the stores t min after the addition of thapsigargin, $Ca^{2+}(t = 0)$ is the Ca^{2+} content in the store immediately before the addition of thapsigargin, λ is the time constant, and $Ca^{2+}(B)$ is a small amount of Ca^{2+} that does not decline during the time measured (the sum of $Ca^{2+}(t = 0)$ and $Ca^{2+}(B)$ is the steady-state Ca^{2+} content at the time of addition of thapsigargin) Analysis was performed using the nonlinear regression computer program InPlot

Materials—Collagenase was purchased from CooperBiomedical Inc (Malvern, PA) Polyethylene glycol (M_r 4000), EGTA, and ruthenium red were obtained from Merck (Darmstadt, Germany) Hyaluronidase, phosphocreatine, and creatine kinase were from Boehringer (Mannheim, Germany) NaATP, bovine serum albumin, Triton X-100, HEEDTA, nitrilotriacetic acid, saponin, phenylmethanesulfonyl fluoride, soybean trypsin inhibitor, and trypan blue were from Sigma Hepes was from Research Organics, Inc (Cleveland, OH) Thapsigargin was from LC Services Corp (Woburn, MA) $^{45}Ca^{2+}$ (20 mCi/ml) was from New England Nuclear (Dreieich, Germany) Ins-1,4,5-P_3 and heparin were generously supplied by Dr P Westerduin (Organon Scientific Development Group, Oss, The Netherlands) All other chemicals were of analytical grade

RESULTS

Isolated rabbit pancreatic acinar cells permeabilized by saponin treatment maximally accumulated 3 03 (S E = 0.22, n = 20) nmol of Ca^{2+}/mg of protein in an energy-dependent Ca^{2+} storage pool when incubated at an ambient free Ca^{2+} concentration of 190 nM Steady-state Ca^{2+} uptake was reached within 10 min (see also Refs 19, 21, and 22), and subsequent addition of Ins-1,4,5-P_3 maximally released 57% (S.E = 3, n = 10) of the actively stored Ca^{2+} Maximal and half-maximal release values were obtained with 10 and 1 μM (S E = 0 1, n = 10) Ins-1,4,5-P_3, respectively

TABLE I

Kinetics of thapsigargin-induced Ca^{2+} loss from intracellular stores

Permeabilized pancreatic acinar cells loaded with Ca^{2+} to steady state were treated with 1 μM thapsigargin At 5 min intervals after thapsigargin addition, the cells were stimulated with 0, 1, or 10 μM Ins-1,4,5-P_3 The reactions were quenched after 0 5, 1 5, and 2 5 min, respectively The residual Ca^{2+} content was calculated, corrected for uptake in the absence of ATP, and expressed as a percentage of the untreated control immediately before thapsigargin addition In each individual experiment, the residual Ca^{2+} contents obtained with 0, 1, or 10 μM Ins-1,4,5-P_3 at the various time points after thapsigargin addition were fitted to the monoexponential function described under "Experimental Procedures" Listed are the means ± S E of (i) the residual Ca^{2+} content of the various pools at the time of thapsigargin addition (t = 0), (ii) the time constant for the thapsigargin-induced loss of Ca^{2+} from the various pools, and (iii) the size of the thapsigargin-insensitive pool (B), calculated from the fits of five individual incubations The effect of thapsigargin on the various pools was followed for 35 min From the time constants, half-times of 6 20 5 60, and 5 25 min were calculated for the decay of the entire energy-dependent pool and for the remainder after stimulation with either 1 or 10 μM Ins-1,4,5-P_3, the latter representing the Ins-1,4,5-P_3-insensitive Ca^{2+} pool The decay of both the Ins 1,4,5-P_3-sensitive pool and the more sensitive part of this pool, releasable by 1 μM Ins-1,4,5-P_3, could be fitted to a monoexponential function Introduction of a constant (B) did not result in a better fit, indicating that both pools decayed in a pure monoexponential fashion

	Ca^{2+}	Time constant	$Ca^{2+}(B)$
	% of control	*min*$^{-1}$	*% of control*
Residual Ca^{2+}			
Control	92 0 ± 2 5	0 112 ± 0 007 ($t_{½}$ = 6 19 min)	8 0 ± 2 5
1 μM Ins-1,4,5-P_3	67 2 ± 2 9	0 124 ± 0 007 ($t_{½}$ = 5 59 min)	8 5 ± 2 2
10 μM Ins-1,4,5-P_3	30 4 ± 2 6	0 132 ± 0 014 ($t_{½}$ = 5 25 min)	7 2 ± 1 8
Releasable Ca^{2+}			
1 μM Ins-1,4,5-P_3	24 7 ± 4 1	0 091 ± 0 008[a] ($t_{½}$ = 7 62 min)	0
10 μM Ins 1,4,5-P_3	62 4 ± 4 1	0 099 ± 0 007[a] ($t_{½}$ = 7 00 min)	0

[a] $p < 0$ 05 as compared with the time constant of the decay of the Ins-1,4,5-P_3 insensitive pool

Kinetics of Ins-1,4,5-P_3 induced Ca^{2+} Release in Absence and Presence of Ca^{2+} Pumping—We have previously shown that thapsigargin, in contrast to vanadate, virtually completely inhibits the exchange of Ca^{2+} in permeabilized acinar cells loaded with Ca^{2+} to steady state (19) This observation indicates that this drug can be used to switch off effectively the Ca^{2+} pump under these experimental conditions The present study shows that the addition of thapsigargin (1 μM) evoked the loss of ~92% of the actively stored Ca^{2+} in a monoexponential manner with an average half-time of 6 2 min (Table I)

To investigate the effect of pump inhibition on the kinetics of Ins-1,4,5-P_3-stimulated Ca^{2+} release, permeabilized acinar cells loaded with Ca^{2+} to steady state were challenged with either a maximal (20 μM) or a submaximal (0 6 μM) concentration of Ins-1,4,5-P_3 at various time points following the addition of 1 μM thapsigargin In each case, the reaction was quenched 6.5 min after the onset of thapsigargin treatment This protocol ensured that pump inhibition affected all release values to the same extent. Fig 1*B* demonstrates that maximal stimulation with Ins-1,4,5-P_3 evokes a rapid release of Ca^{2+} from the Ins-1,4,5-P_3-sensitive store The effect of Ins-1,4,5-P_3 was virtually completed within 1.5 min following the onset of stimulation, indicating that all Ins-1,4,5-P_3-sensitive stores were emptied As expected, the initial release response to a submaximal concentration of Ins-1,4,5-P_3 was less rapid Interestingly, however, the release response did not cease during at least 5 min following the onset of stimulation, indicating that part of the

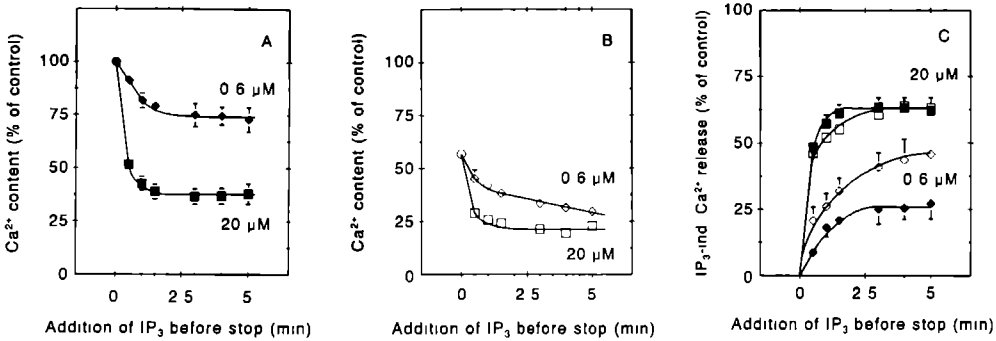

FIG 1 **Effect of Ca^{2+} pump inhibition on kinetics of Ins-1,4,5-P_3-induced Ca^{2+} release.** Permeabilized acinar cells loaded with Ca^{2+} to steady state were treated with either dimethyl sulfoxide (*A*) or 1 μM thapsigargin (*B*) All incubations were stopped at 6 5 min following the onset of thapsigargin treatment Ins-1 4,5-P_3, at final concentrations of 0 6 μM (*diamonds*) and 20 μM (*squares*), was added at the indicated time points before the incubation was stopped The steady-state Ca^{2+} content at 6 5 min following the onset of dimethyl sulfoxide treatment is set at 100%, to which all other values are related The data presented are the means ± S E of three independent experiments In *C*, the release data obtained with the dimethyl sulfoxide-treated (*closed symbols*) and thapsigargin-treated (*open symbols*) cells are expressed as a percentage of the residual Ca^{2+} content of the respective unstimulated controls

Ins-1,4,5-P_3-sensitive store was released at a relatively slow rate in the absence of Ca^{2+} pumping On the other hand, submaximally stimulated Ca^{2+} release from permeabilized cells incubated in the absence of thapsigargin was virtually completed within 1 5 min following the onset of stimulation (Fig 1*A*) The above observations demonstrate that in the presence of pump activity, Ca^{2+} release from relatively slowly releasing stores is compensated for by active Ca^{2+} uptake

Both in the absence and presence of thapsigargin, maximal stimulation resulted in the rapid release of ~63% of the actively stored Ca^{2+} (Fig 1*C*) Fig 1*C*, in which the residual Ca^{2+} content after 6 5 min of incubation in either the presence (*open symbols*) or absence (*closed symbols*) of thapsigargin is set at 100%, shows that thapsigargin markedly increased the apparent ability of Ins-1,4,5-P_3 to release Ca^{2+}, however, without affecting its efficacy Moreover, Fig 1*C* clearly demonstrates the sustained nature of the submaximal release response in the absence of active Ca^{2+} pumping

Inhibition of Ca^{2+} pumping leads to store depletion and, in this way, to reduction of driving force for both passive and Ins-1,4,5-P_3-stimulated Ca^{2+} release To test the possibility that reduction of driving force might have changed the kinetics of Ca^{2+} efflux, experiments were performed in which unidirectional Ca^{2+} flux was studied in the presence of Ca^{2+} pumping After loading to steady state in the presence of a tracer amount of $^{45}Ca^{2+}$, the specific activity of $^{45}Ca^{2+}$ was reduced 28 times, without changing the ambient free Ca^{2+} concentration Fig 2 shows that such a reduction of the specific activity of extravesicular $^{45}Ca^{2+}$ resulted in a monoexponential loss of $^{45}Ca^{2+}$ from the Ins-1,4,5-P_3-sensitive store The addition of a submaximal concentration of Ins-1,4,5-P_3 evoked an instantaneously rapid loss of $^{45}Ca^{2+}$, followed by a sustained phase of increased $^{45}Ca^{2+}$ loss The rate constant of this sustained phase of Ins-1,4,5-P_3-induced $^{45}Ca^{2+}$ loss was clearly dose-dependent This observation demonstrates that in the continuous presence of a submaximal concentration of Ins-1,4,5-P_3, a new steady state is established in the remainder of the Ins-1,4,5-P_3-sensitive store in which the rate of Ca^{2+} pumping is markedly enhanced to compensate for an increased rate of Ca^{2+} loss

A major concern with this type of study is the possibility of Ins-1,4,5-P_3 being rapidly metabolized However, rather than using more stable but less potent Ins-1,4,5-P_3 analogs, which may also display different release properties (24), we preferred

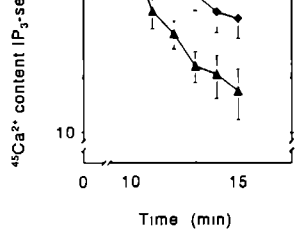

FIG 2 **Effect of Ins-1,4,5-P_3 on kinetics of unidirectional Ca^{2+} flux in presence of Ca^{2+} pumping.** Permeabilized acinar cells were loaded with Ca^{2+} to steady state in the presence of a tracer amount of $^{45}Ca^{2+}$ for 10 min at 37 °C The total chelator concentration in the Ca^{2+} uptake medium was 0 45 mM At 10 min, the suspension was diluted three times with Ca^{2+} uptake medium to which no $^{45}Ca^{2+}$ was added and which contained a total chelator concentration of 4 2 mM To maintain an ambient free Ca^{2+} concentration of 190 nM, total Ca^{2+} had to be markedly increased, resulting in a 28-fold reduction in the specific activity of extravesicular $^{45}Ca^{2+}$ Ins-1,4,5-P_3 at final concentrations of 0, 0 6, 1 2, and 30 μM, was included in the dilution medium The reaction was quenched at the indicated times, and the residual $^{45}Ca^{2+}$ content was measured At each time point, the residual $^{45}Ca^{2+}$ content of the Ins-1,4,5-P_3 sensitive store, calculated from the difference between the contents after stimulation with 0 μM (*closed circles*), 0 6 μM (*closed diamonds*), or 1 2 μM (*closed triangles*) Ins-1,4,5-P_3 and a maximal concentration of 30 μM Ins 1,4,5-P_3, is expressed as a percentage of the maximal $^{45}Ca^{2+}$ content determined immediately before dilution The data presented are the means ± S E of three independent experiments

to use Ins-1,4,5-P_3 itself From the observation that no reuptake of Ca^{2+} was observed over a period of 5 min following the onset of stimulation with a submaximal concentration of 0 6 μM Ins-1,4,5-P_3 (Fig 1*A*), it can be concluded that the Ins-1,4,5-P_3 concentration was not substantially reduced

Apparent Increase in Ins-1,4,5-P_3 Sensitivity during Inhibition of Ca^{2+} Pumping by Thapsigargin—To test the hypothesis that a reduction of the luminal Ca^{2+} content of the Ins-1,4,5-P_3 releasable store would lead to a decreased sensitivity for Ins-1,4,5-P_3, permeabilized cells loaded with Ca^{2+} to steady

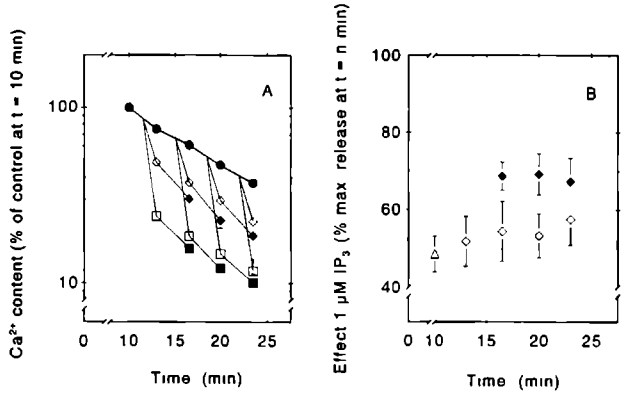

FIG 3 **Effect of decreasing intravesicular Ca^{2+} content on sensitivity of Ins-1,4,5-P_3-releasable Ca^{2+} store to Ins-1,4,5-P_3** *A*, permeabilized acinar cells loaded with Ca^{2+} to steady state were treated with 1 μM thapsigargin at 10 min following the onset of ATP dependent Ca^{2+} loading The cells were stimulated with either 1 μM (*diamonds*) or 20 μM (*squares*) Ins 1,4 5 P_3 at the indicated times The reactions were terminated at either 1 5 min (*open symbols*) or 5 min (*closed symbols*) following Ins 1 4 5-P_3 addition The data presented are the means ± S E of three independent experiments *B* the apparent ability of Ins 1 4 5-P_3 to release Ca^{2+} in the absence of pumping activity is demonstrated by expressing the amount of Ca^{2+} released by the submaximal concentration of Ins 1 4 5 P_3 during 1 5 min (*open diamonds*) and 5 min (*closed diamonds*) as a percentage of the corresponding maximal release value The *open triangle* reflects the percentage of the Ins 1 4,5 P_3 sensitive pool released by 1 μM Ins 1 4 5 P_3 immediately before thapsigargin addition

state were stimulated with Ins-1,4,5 P_3 at different time points following the onset of thapsigargin treatment (Fig 3) In contrast to the protocol used in Fig 1, the reaction was quenched at both 1 5 min (*open symbols*) and 5 min (*closed symbols*) after stimulation with either 0 6 μM (*diamonds*) or 20 μM (*squares*) Ins 1,4,5 P_3 The latter protocol ensured that the release data obtained were not influenced by Ins-1,4,5 P_3 hydrolysis

Fig 3*A* reproduces the observation presented in Fig 1 in that a submaximal concentration of Ins 1,4,5 P_3 evoked an initially rapid release followed by a sustained slow release, whereas the release response evoked by a maximal concentration of Ins-1,4,5-P_3 was already completed within the first 1 5 min of stimulation (note that the scale of the *y* axis is logarithmic) The fractional release evoked by submaximal stimulation, determined at both 1 5 and 5 min following the onset of stimulation, did not change during store depletion (Fig 3*B*) Between 0 and 13 min after the addition of thapsigargin, 0 6 μM Ins-1,4,5-P_3 invariably released ~54% (*open diamonds*) and 68% (*closed diamonds*) of the Ins 1,4,5-P_3-sensitive store within the first 1 5 and 5 min following the onset of stimulation, indicating that the ability of Ins-1,4,5 P_3 to release Ca^{2+} remained unchanged In the presence of pump activity, 0 6 μM Ins-1,4,5-P_3 maximally released only 48% of the Ins-1,4,5-P_3 releasable pool (*open triangle*) This effect was reached within 1 5 min after the onset of stimulation

In the above experiments, the size of the Ins 1,4,5 P_3 sensitive pool after 13 min of thapsigargin treatment was reduced to 41% of its original value To verify that the luminal Ca^{2+} content does not control Ins-1,4,5-P_3 sensitivity at more reduced levels, steady state loaded cells were challenged with both a submaximal and a maximal concentration of Ins 1,4,5-P_3 at 5-min intervals over a period of 35 min The thapsigargin-sensitive part of the Ins-1,4,5-P_3-insensitive Ca^{2+} pool (30% of the residual Ca^{2+} content) decayed in a monoexponential manner with an average half time of 5 25 min (Table I) Although this value tended to be higher than the half-time value of 6 20 min obtained for the energy dependent store as a whole, no statistical significance was reached

The change in size of the most sensitive part of the Ins-1,4,5-P_3-sensitive Ca^{2+} pool was determined by measuring the residual Ca^{2+} content at 1 5 min following submaximal stimulation with Ins-1,4,5-P_3 Approximately 67% of the residual Ca^{2+} content decayed in a monoexponential manner with an average half time of 5 60 min (Table I) Again, 8% of the energy-dependent store did not change in response to thapsigargin

To calculate the releasable amounts of Ca^{2+} at the different time points, the residual Ca^{2+} contents of the control-treated cells were subtracted from those of the Ins 1,4,5-P_3-treated incubations During 35 min of incubation in the presence of thapsigargin, the size of the Ins 1 4,5-P_3-sensitive pool declined to 5% of its original value The decay of the Ca^{2+} content of the Ins 1,4,5-P_3 sensitive Ca^{2+} pool could be described by a monoexponential function with a half-time of 7 00 min (Table I) The calculated time constant was significantly smaller ($p < 0\ 05$) than that obtained for the Ins-1,4,5-P_3-insensitive Ca^{2+} pool, indicating that in the presence of thapsigargin, the Ins-1,4,5 P_3 insensitive Ca^{2+} pool was depleted ~1 3-fold faster than the Ins 1,4,5-P_3 sensitive Ca^{2+} pool The time constant with which the amount of Ca^{2+} releasable by 1 μM Ins-1,4,5-P_3 decayed was not different from that of the Ins 1,4,5 P_3-sensitive Ca^{2+} pool as a whole, confirming that the sensitivity of the Ins-1,4,5 P_3-operated Ca^{2+} release mechanism remained unchanged during depletion of the Ins-1,4,5 P_3 sensitive Ca^{2+} pool

Lack of Effect of Ruthenium Red evoked Store Depletion on Ins 1,4,5 P_3 Sensitivity of Ins 1,4 5 P_3 releasable Ca^{2+} Stores— An alternative approach to establishing a regulatory role for luminal Ca^{2+} in the process of Ins 1,4,5-P_3-stimulated Ca^{2+} release was to deplete preferentially the Ins-1,4,5 P_3-sensitive Ca^{2+} pool by means of ruthenium red We have previously shown that ruthenium red at concentrations below 100 μM predominantly prevents ATP dependent accumulation of Ca^{2+} in the Ins-1,4,5-P_3-sensitive Ca^{2+} pool (19) Table II shows that ruthenium red dose dependently reduced the size of the Ins-1,4,5 P_3 sensitive Ca^{2+} pool In the presence of 70 μM ruthenium red, the size of the Ins 1,4,5-P_3 sensitive Ca^{2+} pool was reduced by ~60%, whereas the same concentration of ruthenium red reduced the size of the Ins 1,4,5-P_3-insensitive Ca^{2+} pool by no more than 20% Subsequent determination of the half-maximal stimulatory concentration of Ins-1 4,5-P_3 revealed that this parameter did not change with the decrease in size of the Ins-

TABLE II
Reduction in size of intracellular Ca^{2+} pools by action of ruthenium red

Permeabilized pancreatic acinar cells were loaded with Ca^{2+} to steady state in the presence of the indicated concentrations of ruthenium red At 10 5 min, either saline or Ins-1,4,5-P, (10 μM) was added, and 1 5 min later, the reaction was quenched After correction for ATP independent Ca^{2+} uptake, actively stored Ca^{2+} in saline-treated cells was set at 100%, to which all other values are related The size of the Ins-1,4,5 P_3 insensitive Ca^{2+} pool is defined as the residual amount of Ca^{2+} after maximal stimulation with 10 μM Ins-1,4,5-P_3 The data presented are the means ± S E of the number of experiments given in parentheses

Ruthenium red conc	Energy dependent Ca^{2+} accumulation	Ins-1,4,5-P_3 insensitive pool	Ins 1,4,5 P_3 sensitive pool
	%	%	%
Control (n = 10)	100	43 ± 3	57 ± 3
20 μM (n = 3)	88 ± 3	43 ± 3	45 ± 4
35 μM (n = 4)	86 ± 6	40 ± 2	46 ± 7
50 μM (n = 8)	66 ± 5	37 ± 3	29 ± 4
70 μM (n = 7)	57 ± 3	34 ± 3	23 ± 3

1,4,5-P_3-sensitive Ca^{2+} pool (Fig 4), indicating that the intravesicular Ca^{2+} concentration did not affect the sensitivity of the Ins-1,4,5-P_3-operated Ca^{2+} release mechanism to Ins-1,4,5-P_3

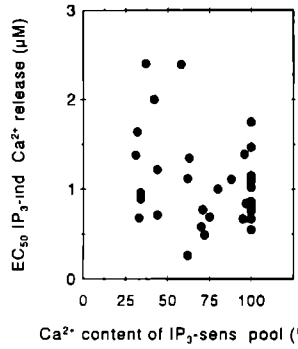

FIG 4 **Lack of effect of luminal Ca^{2+} content of Ins-1,4,5-P_3-sensitive Ca^{2+} pool, reduced by action of ruthenium red, on Ins-1,4,5-P_3 sensitivity.** Permeabilized pancreatic acinar cells were loaded with Ca^{2+} in the absence and presence of ruthenium red at concentrations varying between 20 and 70 μM At 10 min either saline or Ins-1,4,5-P_3 was added, and the reaction was stopped 1 5 min later In each experiment, the size of the Ins-1,4,5-P_3-sensitive Ca^{2+} pool, obtained by maximal stimulation with 10 μM Ins-1,4,5-P_3 in the absence of ruthenium red, is set at 100%, to which all other values are related To prepare the dose-response curve for the Ins-1,4,5-P_3-induced release of Ca^{2+}, aliquots of the cell suspension were stimulated with 0, 0 3, 0 6, 1, 2, 3 3, 5, and 10 μM Ins-1,4,5-P_3, and the release values were used to calculate the half-maximal stimulatory Ins-1,4,5-P_3 concentration as described under "Experimental Procedures"

DISCUSSION

Permeabilized pancreatic acinar cells rapidly accumulated Ca^{2+} in internal stores of nonmitochondrial origin until a steady state was reached between active Ca^{2+} uptake and passive Ca^{2+} loss Assuming that this passive Ca^{2+} leak was not introduced by saponin treatment and that the Ca^{2+} pump was fully active under the experimental conditions used, the steady-state Ca^{2+} content of the energy-dependent loaded stores will be close to physiological values Maximal stimulation with Ins-1,4,5-P_3 revealed that ~60% of the total amount of actively stored Ca^{2+} resided in the Ins-1,4,5-P_3-sensitive store

This study shows that complete inhibition of active Ca^{2+} pumping by thapsigargin resulted in the monoexponential loss of 92% of the steady-state Ca^{2+} content with a half-time of 6 2 min The conclusion that part of the energy-dependent loaded store is insensitive to pump inhibition was also reached by Oldershaw *et al* (4) To investigate the rate of Ca^{2+} loss for the separate internal Ca^{2+} stores, steady-state loaded permeabilized cells were challenged with a maximal concentration of Ins-1,4,5-P_3 at several time points following the onset of pump inhibition From the residual Ca^{2+} content, determined at 2 5 min after stimulation, when the release process was completed, the size of both the Ins-1,4,5-P_3-sensitive and the Ins-1,4,5-P_3-insensitive stores could be calculated Analysis of the thapsigargin-evoked Ca^{2+} loss revealed that both stores decayed in a monoexponential manner The calculated half-times were 7 0 min for the Ins-1,4,5-P_3-sensitive store and 5 3 min for the Ins-1,4,5-P_3-insensitive store, which means that the relative size of the Ins-1,4,5-P_3-sensitive store increased during store depletion By contrast, Marshall and Taylor (13), using permeabilized hepatocytes, recently reported a progressive decrease in the relative size of the Ins-1,4,5-P_3-sensitive store once the Ca^{2+} content of the energy-dependent store, defined as the amount of ionomycin-releasable Ca^{2+}, dropped below 50% of its original value However, this decrease is only apparent since it is due to the fact that the ionomycin-releasable pool was not corrected for the amount of Ca^{2+} that could not be released by thapsigargin and that, in permeabilized hepatocytes, amounted to ~10% of the energy-dependent store (25)

Stimulation with a submaximal concentration of Ins-1,4,5-P_3, which released ~40% of the Ins-1,4,5-P_3-sensitive store in the absence of thapsigargin, at various time points following the onset of pump inhibition revealed that also the most sensitive part of the Ins-1,4,5-P_3-sensitive store decayed in a monoexponential manner The calculated half-time was 7 6 min, which was not significantly different from that of the Ins-1,4,5-P_3-sensitive pool as a whole Since the reaction was quenched at 1 5 min following stimulation as the release response in the absence of active Ca^{2+} pumping was still not complete, the monoexponential nature of the rate of decay of the most sensitive part of the Ins-1,4,5-P_3-releasable store indicates that the kinetics of the release response did not change as a consequence of store depletion The importance of this finding is that it clearly demonstrates that reduction of the Ca^{2+} content of the Ins-1,4,5-P_3-releasable store by 95% did not result in any decrease in Ins-1,4,5-P_3 sensitivity since fitting to a monoexponential function would have been impossible if such a decrease had occurred

The conclusion that reduction of the intravesicular Ca^{2+} content does not affect the sensitivity of the Ins-1,4,5-P_3-sensitive Ca^{2+} store to Ins-1,4,5-P_3 was further substantiated by the finding that a significant reduction by 75% of the Ca^{2+} content of the Ins-1,4,5-P_3-sensitive store by ruthenium red did not result in any decrease in the half-maximal stimulatory concentration of Ins-1,4,5-P_3 Our conclusion that a reduction of luminal Ca^{2+} does not decrease Ins-1,4,5-P_3 sensitivity is in line with earlier studies using permeabilized nasal gland cells (11) and permeabilized hepatocytes (12, 13)

The most intriguing finding of this study is that submaximal concentrations of Ins-1,4,5-P_3 evoked a sustained Ca^{2+} release response in the absence of Ca^{2+} pumping The data obtained in the $^{45}Ca^{2+}/^{40}Ca^{2+}$ exchange experiment demonstrate that the occurrence of such a sustained release response can be explained by the presence of stores in which submaximal Ins-1,4,5-P_3 concentrations evoke a relatively small Ca^{2+} leak, which, in the presence of Ca^{2+} pump activity, is largely compensated for by active Ca^{2+} uptake The initial rapid release response is then explained by stores in which submaximal Ins-1,4,5-P_3 concentrations evoke a large Ca^{2+} leak, which cannot be compensated for by active Ca^{2+} pumping even when pump activity is considerably enhanced as a consequence of store

depletion (24) The observations suggest that whether or not a particular Ca^{2+} storage organelle will be released by a submaximal concentration of Ins-1,4,5-P_3 depends on the ratio between Ins-1,4,5-P_3-stimulated Ca^{2+} release, determined by the number and/or the Ins-1,4,5-P_3 sensitivity of the Ca^{2+} release channels, and Ca^{2+} pump activity, determined by the number and/or the activation state of the Ca^{2+} pumps The simplest structural base for store heterogeneity would be the occurrence of Ca^{2+} storage organelles expressing different amounts of Ins-1,4,5-P_3-operated Ca^{2+} release channels The observation that in the case of maximal stimulation all stores are rapidly depleted then indicates that the maximal opening of only a few channels must already be sufficient to overcome completely compensatory Ca^{2+} pumping On the other hand, the occurrence of Ins-1,4,5-P_3 receptors displaying different sensitivities toward Ins-1,4,5-P_3 can also provide for store heterogeneity Receptors differing in sensitivity then have to be sorted to separate Ca^{2+}-sequestering organelles Evidence in support of the occurrence of store heterogeneity has recently been obtained by means of digital imaging microscopy (26) and confocal laser scan microscopy (27) of pancreatic acinar cells loaded with fluorescent Ca^{2+} indicators

The data presented do not support the idea that intravesicular Ca^{2+} plays a regulatory role in the process of Ins-1,4,5-P_3-induced Ca^{2+} mobilization By contrast, they demonstrate that the quantal nature of Ins-1,4,5-P_3-stimulated Ca^{2+} release, as originally described by Muallem *et al* (1), is most likely explained by store heterogeneity Thus, stimulation with a submaximal concentration of Ins-1,4,5-P_3 rapidly depletes those stores in which it evokes a relatively large Ca^{2+} leak, which cannot be compensated for by active Ca^{2+} uptake, whereas in other stores in which it evokes a smaller Ca^{2+} leak, enhanced Ca^{2+} release can be completely or partially compensated for by increased pumping activity The size of the Ca^{2+} leak, evoked by a submaximal Ins-1,4,5-P_3 concentration, is determined by the number of Ins-1,4,5-P_3-operated Ca^{2+} channels and/or their sensitivity to opening by Ins-1,4,5-P_3

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Chapter 7

Heterogeneous distribution of Ca^{2+} uptake, storage and release sites in permeabilized pancreatic acinar cells

Submitted for publication

Differences in uptake, storage and release properties between inositol trisphosphate-sensitive and -insensitive Ca^{2+} stores in permeabilized pancreatic acinar cells

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ABSTRACT

Rabbit pancreatic acinar cells, permeabilized by saponin treatment, were used to study the kinetics of ATP-dependent Ca^{2+} uptake and release in inositol 1,4,5-trisphosphate-(Ins-1,4,5-P_3)-sensitive and -insensitive stores. Permeabilized acinar cells rapidly accumulated Ca^{2+} to steady-state. At steady state, approximately 60% of actively stored Ca^{2+} resided in the Ins-1,4,5-P_3-sensitive store. Kinetic analysis of the Ca^{2+} uptake process revealed that the initial Ca^{2+} uptake rate was 1.7-times higher in the Ins-1,4,5-P_3-insensitive store as compared to the Ins-1,4,5-P_3-sensitive store. On the other hand, the Ca^{2+} uptake capacity was 1.6-times higher in the Ins-1,4,5-P_3-sensitive store as compared to the Ins-1,4,5-P_3-insensitive store. The Ca^{2+} uptake rate in the Ins-1,4,5-P_3-sensitive store remained constant for at least 4 min, whereas in the Ins-1,4,5-P_3-insensitive Ca^{2+} store this rate progressively declined with time. These observations are compatible with (i) an Ins-1,4,5-P_3-sensitive store containing relatively few Ca^{2+} pumps but possessing a relatively high Ca^{2+} uptake capacity, which may reflect the presence of a substantial amount of Ca^{2+} binding protein, and (ii) an Ins-1,4,5-P_3-insensitive Ca^{2+} store containing relatively many Ca^{2+} pumps but possessing a relatively low Ca^{2+} uptake capacity, which may reflect the presence of little if any Ca^{2+} binding protein. The data presented are consistent with the idea of a heterogeneous distribution of Ca^{2+} pumps, Ca^{2+} binding proteins and Ca^{2+} release channels between intracellular Ca^{2+} storage organelles.

Intracellular Ca^{2+} stores play an important role in cellular Ca^{2+} homeostasis and signal transduction [1]. Many extracellular signals, including hormones, neurotransmitters and physical stimuli, evoke the release of Ca^{2+} from Ca^{2+} storage organelles through the intermediation of inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3), produced upon the receptor-activated hydrolysis of phosphatidylinositol 4,5-bisphosphate.

Digital imaging microscopy has enabled to study the spatial and temporal distribution of such receptor-evoked cytoplasmic Ca^{2+} signals in intact cells. Using this technique, pancreatic acinar cells were demonstrated to respond to receptor activation with a rapidly spreading Ca^{2+} signal, initiated at the secretory pole [2-6]. This initial rise in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was shown to be independent of external Ca^{2+} [2,3,7], indicating that it involves the Ins-1,4,5-P_3-stimulated release of Ca^{2+} from intracellular Ca^{2+} stores. Infusion of a minimal effective concentration of Ins-1,4,5-P_3 at the basolateral side of the acinar cell evoked a Ca^{2+} rise restricted to the apical side of the cell, whereas infusion of a high concentration of Ins-1,4,5-P_3 evoked a global $[Ca^{2+}]_i$ rise [3,5]. In addition, infusion of Ca^{2+} revealed the presence of Ca^{2+}-sensitive Ca^{2+} stores in the apical region of the acinar cell [3]. These observations demonstrate that pancreatic acinar cells possess Ca^{2+} stores with different Ca^{2+} release mechanisms, namely Ca^{2+} stores with Ins-1,4,5-P_3-operated Ca^{2+} release channels and Ca^{2+} stores with Ca^{2+}-activated Ca^{2+} release channels, and that these different Ca^{2+} stores are situated in different areas of the cell. In addition, they demonstrate that Ins-1,4,5-P_3-sensitive Ca^{2+} stores are heterogeneous in their response to Ins-1,4,5-P_3.

Thus far, the mechanism underlying the heterogeneity between Ins-1,4,5-P_3-sensitive Ca^{2+} stores with respect to their releasability by Ins-1,4,5-P_3 is unclear. Basically, there are two explanations for these differences in releasability between internal Ins-1,4,5-P_3-sensitive Ca^{2+} stores. Firstly, Ins-1,4,5-P_3-operated Ca^{2+} release channels may display different sensitivities towards activation by Ins-1,4,5-P_3. It has been demonstrated that alternative splicing leads to the formation of multiple Ins-1,4,5-P_3 receptor subunits and it has been suggested that this may give rise to multiple affinity-binding sites [8]. In addition, the potency of Ins-1,4,5-P_3 in releasing Ca^{2+} has been shown to be altered by phosphorylation of the receptor protein [9-11]. Furthermore, physiological changes in free cytosolic Ca^{2+} concentration [12-15] and cellular pH [16,17] have been demonstrated to control Ins-1,4,5-P_3 binding. Moreover, the intravesicular Ca^{2+} content has been implicated in regulation of the sensitivity of the Ins-1,4,5-P_3-operated Ca^{2+} release channel for activation by Ins-1,4,5-P_3 [18-21]. Secondly, heterogeneity between Ins-1,4,5-P_3-sensitive Ca^{2+} stores can be explained by differences in the relative amount of Ca^{2+} pumps as compared to the amount of Ins-1,4,5-P_3-operated Ca^{2+} release channels [22]. In

the case of a relatively high amount of Ca^{2+} pumps, Ins-1,4,5-P_3-evoked Ca^{2+} release at low Ins-1,4,5-P_3 concentrations will be largely compensated for by active Ca^{2+} uptake, whereas the same Ins-1,4,5-P_3 concentrations will rapidly empty stores possessing relatively few pumps.

In order to gain more insight into the mechanisms underlying heterogeneity between intracellular Ca^{2+} stores, the kinetics of Ca^{2+} uptake and release were studied in permeabilized pancreatic acinar cells. The data obtained are consistent with the idea of a heterogeneous distribution of Ca^{2+} pumps, Ca^{2+} binding proteins and Ca^{2+} release channels between Ins-1,4,5-P_3-sensitive and -insensitive Ca^{2+} storage organelles.

EXPERIMENTAL PROCEDURES

Permeabilization of pancreatic acinar cells

Rabbit pancreatic acinar cells were isolated by enzymatic digestion using collagenase and hyaluronidase as previously described [23]. Acinar cells, permeabilized by saponin treatment, were washed twice and resuspended in a Ca^{2+} uptake medium (4 mg protein/ml) containing 120 mM-KCl, 1.0 mM-$MgCl_2$, 1.2 mM-KH_2PO_4, 5 mM-pyruvate, 5 mM-succinate, 0.5 mM-EGTA, 0.5 mM-nitrilotriacetic acid, 0.5 mM-HEEDTA, 0.2 mg/ml soybean trypsin inhibitor and 20 mM-HEPES and adjusted to pH 7.1 with KOH [22-24]. Permeabilized acinar cells were stored on ice until use.

Ca^{2+} uptake and release experiments

Ca^{2+} uptake was started by adding 10 μl of the permeabilized cell suspension to 90 μl of Ca^{2+} uptake medium which contained in addition: 0 or 1 mM-NaATP, 10 mM creatine phosphate, 10 U/ml creatine kinase, 3% (w/v) polyethylene glycol (Mw. 4000) and 5 μCi $^{45}Ca^{2+}$/ml. The free Mg^{2+} (0.9 mM) and Ca^{2+} (as indicated) concentrations were adjusted as described by Schoenmakers et al. [25]. At 0.5 and 1 μM ambient free Ca^{2+}, 5 μM ruthenium red was included in the medium in order to prevent mitochondrial Ca^{2+} uptake. At this concentration, ruthenium red does not interfere with Ca^{2+} uptake in non-mitochondrial Ca^{2+} stores [24]. The incubations, performed at 37°C, were stopped by adding 1 ml of ice-cold stop solution containing 150 mM-KCl, 5.0 mM-$MgCl_2$, 1.0 mM-EGTA and 20 mM HEPES/KOH (pH 7.1) and the suspension was rapidly filtered (Schleicher and Schüll, GF92, Dassel, Germany). The filters were washed twice and counted for radioactivity. Total Ca^{2+} was calculated and expressed as nmol Ca^{2+} per mg of protein. Actively stored Ca^{2+} is defined as the difference in total Ca^{2+} retained on the filter after incubation in the presence and absence of ATP.

Protein was determined with a commercial Coomassie Blue kit (Bio-Rad) after

treatment of the cells with 0.1% Triton X-100. γ-Globulin (Bio-Rad) was used as a standard.

Ca^{2+} exchange experiments under steady-state conditions

Permeabilized acinar cells were loaded with Ca^{2+} in the presence of radioactive tracer in Ca^{2+} uptake medium in which the concentration of each of the divalent cation chelators was reduced to 0.15 mM. The ambient free Ca^{2+} concentration was adjusted to 0.19 μM. After 10 min of Ca^{2+} loading, the Ca^{2+} uptake medium was diluted 3-fold with medium in which the concentration of each of the chelators was increased to 2 mM and which did not contain $^{45}Ca^{2+}$. The ambient free Ca^{2+} concentration was maintained at 0.19 μM. This procedure resulted in a 28-fold reduction in specific activity of $^{45}Ca^{2+}$, thus allowing to study unidirectional Ca^{2+} fluxes under steady-state conditions.

In a second type of exchange experiment, permeabilized acinar cells were loaded with Ca^{2+} to steady-state in the absence of radioactive tracer. After 10 min of Ca^{2+} loading, $^{45}Ca^{2+}$ was added and accumulated Ca^{2+} was determined at the indicated times.

Analysis of the data

The kinetics with which Ca^{2+} was accumulated in the Ins-1,4,5-P_3-sensitive and -insensitive stores was analysed by means of the nonlinear regression computer program InPlot (GraphPad Software for Science, San Diego, CA).

Materials

Collagenase was purchased from Cooper Biomedical Inc., Malvern, PA. Polyethylene glycol (Mw. 4000), EGTA and ruthenium red were obtained from Merck, Darmstadt, Germany; hyaluronidase, phosphocreatine and creatine kinase were from Boehringer, Mannheim, Germany; NaATP, bovine serum albumin, Triton X-100, HEEDTA, nitrilotriacetic acid, saponin, phenylmethanesulphonyl fluoride, soya-bean trypsin inhibitor and trypan blue were from Sigma, St. Louis, MO.; Hepes was from Research Organics Inc., Cleveland, OH.; thapsigargin was from LC Services Corporation, Woburn, MA.; $^{45}Ca^{2+}$ (20 mCi/ml) was from New England Nuclear, Dreieich, Germany. Ins(1,4,5)P_3 and heparin were generously supplied by Dr. P. Westerduin, Organon Scientific Development Group, Oss, The Netherlands. All other chemicals were of analytical grade.

Time-dependence of ATP-dependent Ca^{2+} uptake in Ins-1,4,5-P_3-sensitive and -insensitive Ca^{2+} stores

Pancreatic acinar cells, permeabilized by saponin treatment, maximally accumulated 2.77 (S.E.= 0.27, n=24) nmol Ca^{2+}/mg of protein in an energy-dependent Ca^{2+} storage pool when incubated at an ambient free Ca^{2+} concentration of 190 nM and a temperature of 37°C. Steady-state Ca^{2+} uptake was reached within 7 min following the addition of ATP **(Fig.1A)** and subsequent addition of Ins-1,4,5-P_3 maximally released 65% of actively stored Ca^{2+}. In order to study the kinetics of the Ca^{2+} uptake process in individual Ca^{2+} stores, Ca^{2+} uptake experiments were performed in the presence of a maximally effective concentration of Ins-1,4,5-P_3. **Figure 1A** shows that under these conditions steady-state Ca^{2+} uptake reached a maximum of 1.20 nmol Ca^{2+}/mg of protein (S.E.=0.15, n=4) within 3 min following the addition of ATP. Steady-state Ca^{2+} uptake remained maximal for another 7 min, indicating that no significant Ins-1,4,5-P_3 degradation occurred. The steady-state level attained in the presence of a maximally effective concentration of Ins-1,4,5-P_3 was virtually similar to that reached 2 min after maximal stimulation of steady-state loaded control cells **(Fig.1C, open triangle)**.

Non-linear regression analysis revealed that the uptake process in this store occurred according to a monoexponential rate equation with a half-time of 1.1 min^{-1} **(Fig.1C)**. The initial Ca^{2+} uptake rate in the Ins-1,4,5-P_3-insensitive store was calculated to be 0.79 nmol Ca^{2+}/mg protein.min^{-1}. The time-dependence of the Ca^{2+} uptake in the Ins-1,4,5-P_3-sensitive store was estimated by subtracting the Ca^{2+} uptake values measured in the presence of Ins-1,4,5-P_3 from those measured in the absence of Ins-1,4,5-P_3. **Figure 1B** shows that Ca^{2+} uptake in the Ins-1,4,5-P_3-sensitive store did not reach steady-state before 7 min following the addition of ATP. Ca^{2+} uptake was virtually linear with time during the first 4 min and could therefore by no means be fitted to a monoexponential rate equation. Linear regression analysis revealed an initial Ca^{2+} uptake rate of 0.28 nmol Ca^{2+}/mg of protein.min^{-1}.

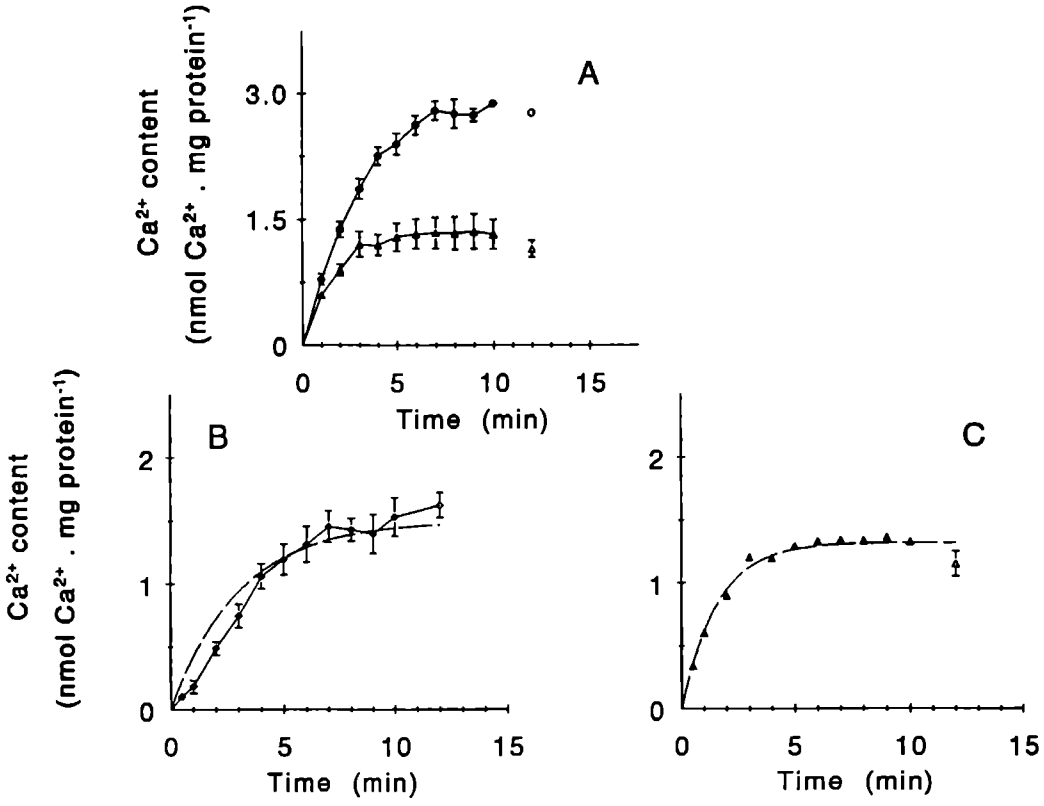

Figure 1. *Time-dependence of active Ca^{2+} uptake in Ins-1,4,5-P_3-sensitive and -insensitive intracellular Ca^{2+} stores.* Permeabilized pancreatic acinar cells were loaded with Ca^{2+} in the absence (closed circles) or presence (closed triangles) of a maximally effective concentration of Ins-1,4,5-P_3 (30 μM). The ambient free Ca^{2+} concentration was 0.19 μM and the incubations were performed at 37°C. The reactions were terminated at the indicated times. The open symbols represent the residual Ca^{2+} content in control cells loaded with Ca^{2+} for 10 min and subsequently incubated in the presence of either saline (open circle) or 30 μM Ins-1,4,5-P_3 (open triangle) for another 2 min. The residual Ca^{2+} content was calculated and corrected for uptake in the absence of ATP. The values presented are the mean ± S.E. of at least four independent experiments. **(A)** Time-dependence of Ca^{2+} uptake in the absence (closed circles) and presence (closed triangles) of 30 μM Ins-1,4,5-P_3. **(B)** Time-dependence of Ca^{2+} uptake in the Ins-1,4,5-P_3-sensitive store. The dashed line represents a tentative fit of the Ca^{2+} uptake process to a monoexponential rate equation. The fit was obtained by fixating the maximal Ca^{2+} uptake value. **(C)** Time-dependence of Ca^{2+} uptake in the Ins-1,4,5-P_3-insensitive store. The dashed line represents the fit of the Ca^{2+} uptake process to a monoexponential rate equation. The fit was obtained without fixating the maximal Ca^{2+} uptake value.

Ca^{2+}-dependence of steady-state ATP-dependent Ca^{2+} uptake in Ins-1,4,5-P_3-sensitive and -insensitive Ca^{2+} stores

The steady-state Ca^{2+} uptake level dose-dependently increased with increasing of the ambient free Ca^{2+} concentration (**Fig.2**). At 1.0 μM free Ca^{2+}, steady-state Ca^{2+} uptake reached a value 1.3-fold higher (3.57 nmol Ca^{2+}/mg of protein (S.E.=0.21, n=3) than that obtained at an ambient free Ca^{2+} concentration of 0.19 μM. In relative terms, the size of the Ins-1,4,5-P_3-sensitive store, determined by challenging steady-state loaded cells with a maximally effective concentration of Ins-1,4,5-P_3 (20 μM), did not change upon increasing of the ambient free Ca^{2+} concentration (see also, Ref.[26]). At 0.19 μM, 0.5 μM and 1.0 μM $[Ca^{2+}]_{free}$, 20 μM Ins-1,4,5-P_3 released 63% (1.73 nmol Ca^{2+}/mg of protein (S.E.=0.05, n=3)), 64% (2.01 nmol Ca^{2+}/mg of protein (S.E.=0.06, n=3)) and 62% (2.21 nmol Ca^{2+}/mg of protein (S.E.=0.17, n=3)) of actively stored Ca^{2+}, respectively.

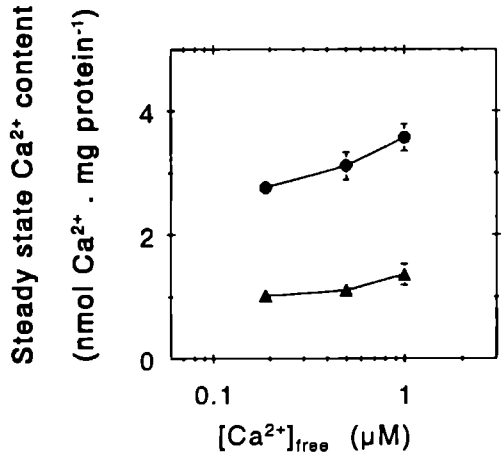

Figure 2. *Dependence of the size of the Ins-1,4,5-P_3-sensitive and -insensitive intracellular Ca^{2+} store on the ambient free Ca^{2+} concentration.* Permeabilized pancreatic acinar cells were loaded with Ca^{2+} to steady-state at the indicated ambient free Ca^{2+} concentrations for 10 min. At 10 min, either saline (closed circles) or a maximally effective concentration of 20 μM Ins-1,4,5-P_3 (closed triangles) was added and the incubations were terminated 2 min later. Actively stored Ca^{2+} is expressed in nanomoles of Ca^{2+} per milligram of acinar protein. The results presented are the mean $\pm$ S.E. of at least three independent experiments, each of which performed in triplicate.

Ca^{2+}-dependence of initial ATP-dependent Ca^{2+} uptake rates in Ins-1,4,5-P_3-sensitive and -insensitive Ca^{2+} stores

In order to assure linearity of ATP-dependent Ca^{2+} uptake with time in experiments aimed to study the Ca^{2+}-dependence of ATP-dependent Ca^{2+} uptake, incubations were terminated after 1 min in the case of an ambient free Ca^{2+} concentration of 0.19 μM and after 0.5 min in the case of 0.5 μM and 1.0 μM free Ca^{2+} **(see, Fig.1A, closed circles)**. At 0.19 μM free Ca^{2+}, permeabilized acinar cells accumulated 0.88 nmol Ca^{2+} per mg of protein per min (S.E.=0.04, n=3) **(Fig.3)**. This value increased with increasing of the ambient free Ca^{2+} concentration to reach a value of 2.02 nmol Ca^{2+} per mg of protein per min (S.E.=0.05, n=3) at 1.0 μM free Ca^{2+}. When initial Ca^{2+} uptake was studied in the presence of a maximally effective concentration of Ins-1,4,5-P_3 (20 μM), reflecting ATP-dependent Ca^{2+} uptake in the Ins-1,4,5-P_3-insensitive store, a value of 0.55 nmol Ca^{2+} per mg of protein per min (S.E.=0.01, n=3) was obtained at 0.19 μM free Ca^{2+}. At ambient free Ca^{2+} concentrations of 0.5 μM and 1.0 μM, the initial Ca^{2+} uptake rates were 0.90 (S.E.=0.13, n=3) and 1.34 nmol Ca^{2+}/mg of protein.min^{-1}, respectively. Calculation of the Ca^{2+} uptake rates for the Ins-1,4,5-P_3-sensitive store yielded values of 0.33, 0.54 and 0.68 nmol Ca^{2+}/mg of protein.min^{-1} at 0.19, 0.5 and 1.0 μM free Ca^{2+}, respectively.

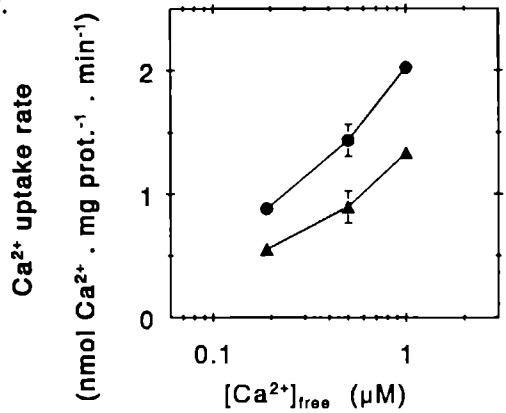

Figure 3. *Dependence of the initial Ca^{2+} uptake rate in the Ins-1,4,5-P_3-sensitive and -insensitive Ca^{2+} store on the ambient free Ca^{2+} concentration.* Permeabilized acinar cells were loaded with Ca^{2+} at the indicated ambient free Ca^{2+} concentrations in the absence (closed circles) or presence (closed triangles) of a maximally effective concentration of 20 μM Ins-1,4,5-P_3. In order to assure linearity of the uptake process, incubations were stopped after 1 min when performed at an ambient free Ca^{2+} concentration of 0.19 μM and after 30 s when performed at ambient free Ca^{2+} concentrations of 0.5 μM and 1.0 μM. Actively stored Ca^{2+} is expressed in nanomoles of Ca^{2+} per milligram of acinar protein. The results are the mean $\pm$ S.E. of at least three independent experiments, each of which performed in triplicate.

Heparin-induced Ca^{2+} reuptake in depleted Ins-1,4,5-P_3-sensitive Ca^{2+} stores

Addition of heparin to permeabilized pancreatic acinar cells, in which the Ins-1,4,5-P_3-sensitive store was completely depleted by the action of a maximally effective concentration of Ins-1,4,5-P_3, resulted in a rapid reuptake of Ca^{2+} **(Fig.4A)**. This procedure allowed to estimate the Ca^{2+} uptake rate in the Ins-1,4,5-P_3-sensitive store without interference of the Ins-1,4,5-P_3-insensitive store. The Ca^{2+} reuptake rate was calculated to be 0.43 nmol Ca^{2+}/mg of protein.min^{-1}. This value correlated well with that observed in the Ca^{2+} uptake experiments **(Figs. 1B and 3)**. The heparin-induced reuptake of Ca^{2+} was completely prevented by thapsigargin, demonstrating the exclusive involvement of intracellular Ca^{2+}-ATPases.

The effect of decreasing the luminal Ca^{2+} content of the Ins-1,4,5-P_3-sensitive Ca^{2+} pool on the Ca^{2+} pump activity was studied in a $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange experiment. Permeabilized acinar cells were loaded with Ca^{2+} to steady-state in the absence of $^{45}Ca^{2+}$. At 9.5 min either saline or Ins-1,4,5-P_3 (10 μM) was added followed at 10 min by a tracer amount of $^{45}Ca^{2+}$. In the presence of 10 μM Ins-1,4,5-P_3, when the Ins-1,4,5-P_3-sensitive Ca^{2+} pool was maximally depleted, the rate of labeling of the remainder of the energy-dependent store was markedly reduced as compared to the control **(Fig.4B)**. Subsequent addition of heparin (150 U/ml) led to a rapid increase in the rate of labeling, reflecting the increased uptake of Ca^{2+} by the Ins-1,4,5-P_3-sensitive Ca^{2+} pool. Interestingly, the initial rate of labeling was not different between control and Ins-1,4,5-P_3-treated cells. It should be noted that the Ca^{2+} uptake values presented in **figure 4B** are not corrected for those obtained in the absence of ATP.

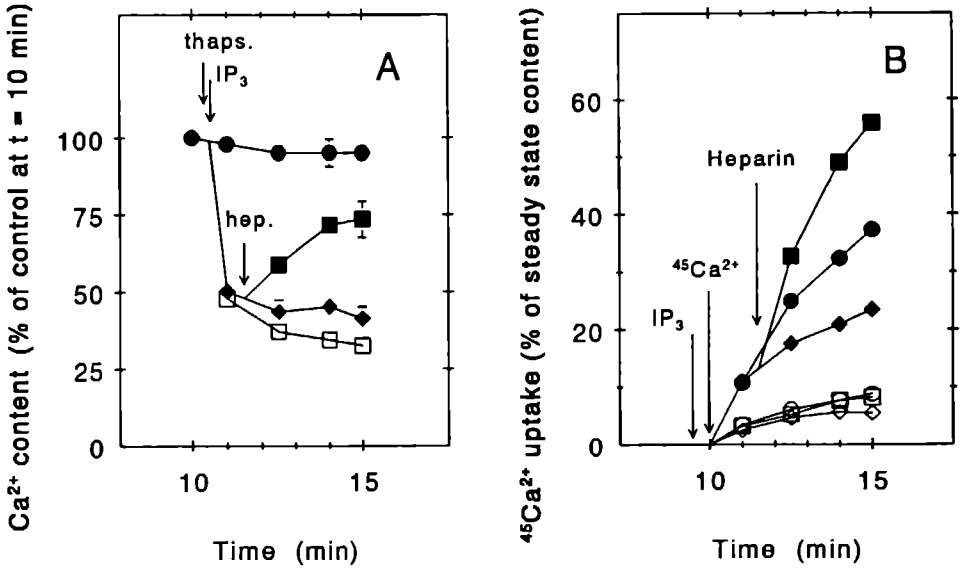

Figure 4. *Heparin-evoked reuptake of Ca^{2+} in Ins-1,4,5-P_3-depleted Ca^{2+} stores.* Permeabilized pancreatic acinar cells, loaded with Ca^{2+} to steady-state in the presence **(A)** or absence **(B)** of radioactive tracer, were treated with either dimethylsulfoxide (closed symbols) or 1 μM thapsigargin (open symbols). **A.** Thapsigargin (open squares) was added at 10.25 min. At 10.5 min either saline (closed circles) or 10 μM Ins-1,4,5-P_3 (open and closed squares and closed diamonds) was added, followed at 11.5 min by the addition of 150 U of heparin/ml (open and closed squares). The reactions were stopped at the indicated times and the residual Ca^{2+} content was determined. The residual Ca^{2+} content at 10 min is set at 100%, to which all other values are related. The data presented are the mean $\pm$ S.E. of 3 independent determinations. **B.** Thapsigargin (open symbols) was added at 9 min. At 9.5 min either saline (circles) or 10 μM Ins-1,4,5-P_3 (squares and diamonds) was added, followed at 11.5 min by the addition of 150 U of heparin/ml (squares). A tracer amount of $^{45}Ca^{2+}$ was added at 10 min. The reactions were stopped at the indicated times and the amount of $^{45}Ca^{2+}$ accumulated under the various experimental conditions is expressed as percentage of the steady-state $^{45}Ca^{2+}$ content. The data presented are from a single experiment which is representative for two independent experiments.

Exchange of $^{45}Ca^{2+}$ under steady-state conditions

In order to study the rate at which Ca^{2+} was lost from permeabilized acinar cells loaded to steady-state in the presence of a tracer amount of $^{45}Ca^{2+}$, the specific activity of the tracer was reduced 28-times without changing the ambient free Ca^{2+} concentration. **Figure 5A** shows that under conditions of steady-state Ca^{2+} loading, 30% of actively stored $^{45}Ca^{2+}$ was lost within the first minute after reduction of the specific activity. Thereafter, $^{45}Ca^{2+}$ was lost in a monoexponential manner. Simultaneous addition of a maximally effective concentration of Ins-1,4,5-P_3, which under normal conditions released 62% of the steady-state Ca^{2+} content, resulted in a loss of 90% of actively stored $^{45}Ca^{2+}$ within the first minute after reduction of the specific activity. The latter observation suggests that the large decrease of the $^{45}Ca^{2+}$ content observed during the first minute of the exchange experiments performed in the absence of Ins-1,4,5-P_3 is mainly due to a high turn-over rate of the Ins-1,4,5-P_3-insensitive pool. The $^{45}Ca^{2+}$ content of the Ins-1,4,5-P_3-sensitive store decayed in a monoexponential fashion with a half-time of 6.5 min (time constant = 0.11 min^{-1}) **(Fig.5B)**. In a previous study, using thapsigargin to study the rate of Ca^{2+} loss, the Ca^{2+} content of the Ins-1,4,5-P_3-insensitive Ca^{2+} store was found to decay with a half-time of 7.0 min [22]. The passive Ca^{2+} permeability of the Ins-1,4,5-P_3-insensitive store was considerably higher since exchange was virtually completed within 1 min **(Fig.5A)**. If the exchange process had occurred with the same time-constant as observed for the Ins-1,4,5-P_3-sensitive store, the $^{45}Ca^{2+}$ content of Ins-1,4,5-P_3-insensitive store would have been reduced by 10% rather than 30% per minute.

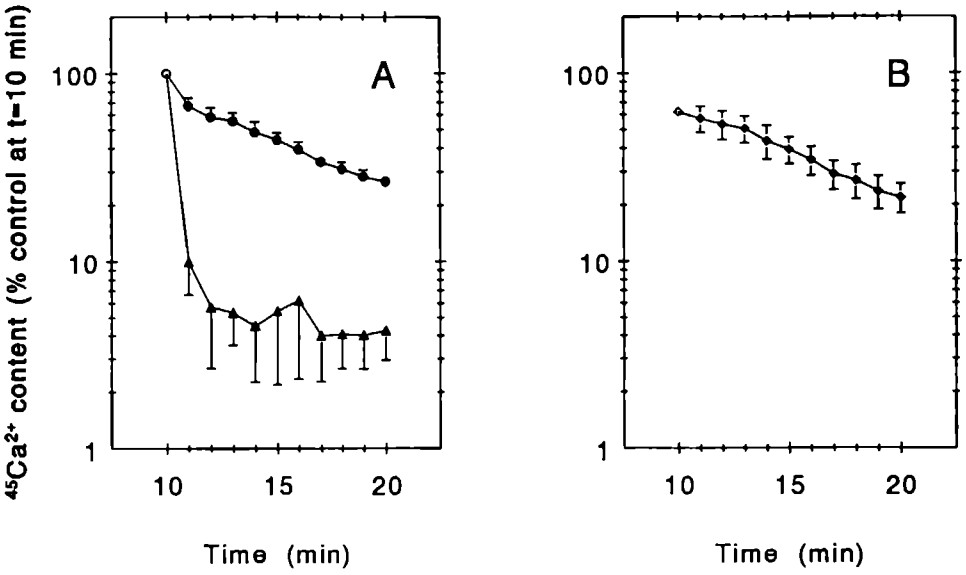

Figure 5. *Effect of Ins-1,4,5-P_3 on the unidirectional Ca^{2+} efflux in the presence of Ca^{2+} pumping.* **(A)** Permeabilized cells were loaded with Ca^{2+} at an ambient free Ca^{2+} concentration of 0.19 μM in a medium containing 0.15 mM of each of the three bivalent cation chelators. After 10 min, the concentration of the radioactive tracer was reduced 28-fold without changing the ambient free Ca^{2+} concentration as described in the experimental procedures. Saline (closed circles) or Ins-1,4,5-P_3 (closed triangles), at a final concentration of 30 μM, was included into the dilution medium. The reactions were quenched at the indicated times. The residual $^{45}Ca^{2+}$ content was corrected for the ATP-independent binding, which was determined in paralel incubations performed in the absence of ATP. The values presented are expressed as percentage of the residual amount of radioactive tracer present at 10 min (open circle). The results presented are the mean $\pm$ S.E. of three independent experiments. **(B)** Represents the exhange activity in the Ins-1,4,5-P_3-sensitive store. The data presented in **(B)** are calculated from the data presented in **(A)**.

The data presented in this study demonstrate that internal Ca^{2+} stores, normally discriminated by virtue of their releasability by Ins-1,4,5-P_3, differ also in a number of other aspects.

Firstly, from the observation that the initial Ca^{2+} uptake rate measured in the presence of a maximally effective concentration of Ins-1,4,5-P_3, thus reflecting active Ca^{2+} uptake in the Ins-1,4,5-P_3-insensitive store, was 1.7-times higher than that calculated for the Ins-1,4,5-P_3-sensitive store it can be deduced that the Ins-1,4,5-P_3-insensitive store contains 1.7-times more Ca^{2+} pumps than the Ins-1,4,5-P_3-sensitive store. Furthermore, from the observation that increasing of the ambient free Ca^{2+} concentration from 0.19 μM to 1.0 μM led to an increase in Ca^{2+} uptake rate which was not significantly different between both stores it can be concluded that both subpopulations of Ca^{2+} pumps are equally sensitive to activation by ambient free Ca^{2+}. However, it can not be ruled out that the differences in initial Ca^{2+} uptake rate, observed in the present study, reflect differences in turnover rate among the two subclasses of Ca^{2+} pumps. Such differences may be intrinsic to the pumping protein itself or the result of a kinase-dependent phosphorylation reaction. Another possibility is the development of a membrane potential opposing ATP-dependent Ca^{2+} uptake, which may be different between both stores.

Secondly, the uptake capacity of the Ins-1,4,5-P_3-sensitive store was 1.6-times higher than that of the Ins-1,4,5-P_3-insensitive store. With increasing of the ambient free Ca^{2+} concentration, the steady-state Ca^{2+} content of both stores increased to the same extent as was revealed by the observation that a maximally effective concentration of Ins-1,4,5-P_3 invariably released 60% of actively stored Ca^{2+}.

Thirdly, Ca^{2+} uptake in the Ins-1,4,5-P_3-sensitive store was virtually linear with time during the first 4 min following its initiation. By contrast, the rate at which Ca^{2+} was actively accumulated in the Ins-1,4,5-P_3-insensitive store decreased progressively with time. This observation may be explained by the presence of substantial amounts of a Ca^{2+} binding protein in the Ins-1,4,5-P_3-sensitive store. This conclusion is supported by recent immunolocalization studies [27]. Using antibodies directed against calsequestrin, a Ca^{2+} binding protein present in the terminal cisternae of the sarcoplasmic reticulum, a calsequestrin-like protein was demonstrated to be present in discrete organelles, referred to as calciosomes. Recently, this protein has been identified as calreticulin [28]. Evidence in support of the co-localization of this Ca^{2+} binding protein and the Ca^{2+} pump was obtained in studies using antibodies raised against the sarcoplasmic reticulum Ca^{2+}-ATPase [29]. Both antibodies were demonstrated to recognize proteins present in small cytosolic structures distributed throughout the acinar cell. Whether these particular structures also contain the Ins-1,4,5-P_3 receptor is still unclear. Interestingly, however, subcellular fractionation studies using canine pancreatic homogenates revealed that the

distribution pattern of the Ins-1,4,5-P_3 receptors was significantly different from that of Ca^{2+} pumps and Ca^{2+} binding proteins [30,31]. This observation is consistent with the data obtained in the present study, suggesting a heterogeneous distribution of Ca^{2+} pumps and Ins-1,4,5-P_3-operated Ca^{2+} release channels between intracellular Ca^{2+} storage organelles. However, it can not be ruled out that the greater extent of linearity of Ca^{2+} uptake, observed for the Ins-1,4,5-P_3-sensitive store in the present study, reflects a larger mean vesicle size, a slower development of an inhibitory membrane potential, or a lower sensitivity of pump to inhibition by intravesicular Ca^{2+}.

Fourthly, the turn-over rate of Ca^{2+} in the Ins-1,4,5-P_3-insensitive store was found to be extremely high. This was concluded from the observation that under steady-state conditions the Ins-1,4,5-P_3-insensitive store lost 80% of its actively stored $^{45}Ca^{2+}$ within the first minute following a 28-fold decrease in specific activity of the radioactive tracer in the medium. Similarly, permeabilized acinar cells, loaded with Ca^{2+} to steady-state in the absence of $^{45}Ca^{2+}$ and stimulated with a maximally effective concentration of Ins-1,4,5-P_3 30 s before the addition of $^{45}Ca^{2+}$ to the medium, accumulated $^{45}Ca^{2+}$ at a rate equal to that obtained with unstimulated cells during the first minute of incubation in the presence of the radioactive tracer. These observations suggest that Ca^{2+}, actively taken up in this store, may largely be present in the unbound form.

In contrast to the Ins-1,4,5-P_3-insensitive store, the Ins-1,4,5-P_3-sensitive store is exchanging more slowly. This is in agreement with the idea that the passive Ca^{2+} permeability of the Ins-1,4,5-P_3-sensitive store is considerably less than that of the Ins-1,4,5-P_3-insensitive store. From the observation that the thapsigargin-evoked loss of Ca^{2+} from the Ins-1,4,5-P_3-sensitive store is a first-order process it can be concluded that the free Ca^{2+} concentration in this store is gradually decreasing during passive Ca^{2+} efflux. This is consistent with the idea that virtually all Ca^{2+}, actively taken up in the Ins-1,4,5-P_3-sensitive store, is loosely bound to Ca^{2+} binding proteins and rapidly exchangeable with Ca^{2+} present in the unbound Ca^{2+} state. In the case of Ca^{2+} being tightly bound, pump inhibition would have led to a considerably slower and linear rather than monoexponential efflux of Ca^{2+}, as has been observed in stores loaded with Ca^{2+} precipitating anions [32].

The use of Ca^{2+} precipitating anions such as oxalate, with a dissociation constant for Ca^{2+} of about 1 mM [33], demonstrates that the intravesicular free Ca^{2+} concentration is in the millimolar range. Recently, millimolar Ca^{2+} concentrations have been measured in intracellular Ca^{2+} stores of permeabilized hepatocytes using the fluorescent Ca^{2+} selective probe chlortetracycline [34]. This is consistent with a functional role for Ca^{2+} sequestering proteins, such as calreticulin, which have been shown to bind Ca^{2+} with affinities in the millimolar range [28].

From the experimental data obtained with the permeabilized cell system, the picture emerges that the pancreatic acinar cell contains essentially two distinct intracellular Ca^{2+} stores of non-mitochondrial origin. One store, containing relatively few Ca^{2+} pumps

but displaying a high Ca^{2+} uptake capacity, possibly due to the presence of a Ca^{2+} binding protein, and released by the action of Ins-1,4,5-P_3, and a second store containing relatively many Ca^{2+} pumps but displaying a low Ca^{2+} uptake capacity and not released by the action of Ins-1,4,5-P_3.

Putney and co-workers have postulated that both Ca^{2+} pumps and Ins-1,4,5-P_3-operated Ca^{2+} release channels reside in the same Ca^{2+} storage organelle but are spatially separated. Permeabilization of the cells could then lead to the formation of smaller organelles containing either Ca^{2+} pumps and little if any Ins-1,4,5-P_3-operated Ca^{2+} release channels or Ins-1,4,5-P_3-operated Ca^{2+} release channels and little if no Ca^{2+} pumps [35], whereas subcellular fractionation could lead to the formation of microsomal vesicles containing either Ins-1,4,5-P_3-operated Ca^{2+} release channels and no Ca^{2+} pumps or Ca^{2+} pumps and no Ins-1,4,5-P_3-operated Ca^{2+} release channels [36]. These observations demonstrate that the possibility of the heterogeneity between Ca^{2+} storage organelles observed in the present study being caused by permeabilization of the cells can not be ruled out.

In a previous study [22], we have postulated that the Ins(1,4,5)$_3$-sensitive store consists of a heterogeneous population of discrete Ca^{2+} accumulating organelles containing different amounts of Ca^{2+} pumps relative to Ins-1,4,5-P_3-operated Ca^{2+} release channels. Ins-1,4,5-P_3-sensitive stores displaying relatively few Ca^{2+} pumps are more sensitive to Ins-1,4,5-P_3 due to a low compensatory Ca^{2+} pumping activity and their presence may therefore be restricted to that part of the apical pole of the acinar cell where the initial Ca^{2+} rise occurs and which has been referred to as 'trigger zone' [3]. On the other hand, Ins-1,4,5-P_3-sensitive stores containing relatively many Ca^{2+} pumps are less sensitive to Ins-1,4,5-P_3 due to a high compensatory Ca^{2+} pumping activity and may therefore be situated in the basolateral area of the acinar cell. Thus far, it is unclear which store contains the Ca^{2+}-activated Ca^{2+} release channel. However, it is temptative to speculate that it is the Ins-1,4,5-P_3-insensitive store, which may then be situated in apical area of the cell, where Ca^{2+} infusion has been demonstrated to initiate a heparin-insensitive rise in free cytosolic Ca^{2+} concentration [3].

Acknowledgments: The research of Dr. P.H.G.M. Willems has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

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Chapter 8

Summary and general discussion

Exocrine pancreatic acinar cells are extensively used as a model to study mechanisms of signal transduction [Gardner and Jensen 1993; Petersen 1993; Williams and Yule 1993]. Stimulation of these cells by the gut hormone CCK or the neurotransmitter ACh results in an enhanced enzyme and fluid secretion. Both CCK and ACh bind to receptors on the basolateral membrane and activate via a guanine nucleotide binding protein (G-protein) phospholipase C activity. Phospholipase C generates the formation of two second messengers by catalysing the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$). The lipophilic messenger DAG stays in the plasmamembrane where it activates protein kinase C, whereas the hydrophilic messenger $Ins(1,4,5)P_3$ diffuses into the cytosol where it releases Ca^{2+} from Ca^{2+} storage organelles, leading to an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and a consequent change in activity of a wide variety of Ca^{2+}-regulated processes. Both pathways result in the phosphorylation of many proteins and have been demonstrated to be necessary in the activation of the mechanisms leading to enzyme and fluid secretion.

Maximal secretagogue stimulation of individual acinar cells results in a rapid and transient elevation of the cytosolic Ca^{2+} concentration from a value of around 0.1 μM to a value of 0.5 to 1.0 μM [Petersen 1993; Williams and Yule 1993]. After this transient rise cytosolic Ca^{2+} returns to a concentration slightly above the resting concentration, provided that the secretagogue receptor remains occupied by its agonist. The initial rise in cytosolic Ca^{2+} virtually completely depends on Ca^{2+} originating from intracellular Ca^{2+} stores, whereas the influx of extracellular Ca^{2+} is needed to maintain the slightly elevated cytosolic Ca^{2+} level during agonist stimulation.

Submaximal stimulation of individual cells results in repetitive and transient rises in cytosolic Ca^{2+}. Also this Ca^{2+} behaviour mainly depends on Ca^{2+} released from intracellular Ca^{2+} sources, whereas extracellular Ca^{2+} is needed for their maintenance. Ca^{2+} oscillations have been implicated in the control of enzyme and fluid secretion in exocrine pancreatic acinar cells. The mechanism explaining Ca^{2+} oscillations is, however, poorly understood. Video-imaging microscopy has revealed that receptor-evoked rises in $[Ca^{2+}]_i$ start at the apical pole and subsequently spread to basolateral areas of the acinar cell. Spreading of the Ca^{2+} signal is thought to occur by a mechanism referred to as Ca^{2+}-induced Ca^{2+} release. It is unclear if this Ca^{2+}-induced Ca^{2+} release mechanism is mediated by Ca^{2+} sensitizing $Ins(1,4,5)P_3$-receptors or by activating ryanodine receptors [Kasai and Augustine 1990; Nathanson et al. 1992; Sharp et al. 1992; Gromada et al. 1993; Kasai et al. 1993; Thorn et al. 1993]. These findings, but also fractionation and morphological studies, indicate the presence of a heterogeneous population of Ca^{2+} stores

in pancreatic acinar cells [Volpe et al. 1988; Hashimoto et al. 1988; Dehlinger-Kremer et al. 1989; Sharp et al. 1992]. In agreement with these findings, this thesis provides evidence for the existence of a heterogeneous population of Ca^{2+} stores in permeabilized acinar cells (see below).

A useful method to study intracellular Ca^{2+} stores is the permeabilized cell system. With this system, in which the plasmamembrane is selectively permeabilized by, for instance, electroporation or treatment with detergents, such as saponin, digitonin or steptolycin-O, easy access is gained to intracellular compartments. Permeabilized cells have the advantage that the cytosolic environment is well defined and that compounds, which normally do not enter the cell, can now be studied. By using this system it was demonstrated for the first time that $Ins(1,4,5)P_3$ releases Ca^{2+} from non-mitochondrial Ca^{2+} stores in pancreatic acinar cells [Streb et al. 1983]. The importance of $Ins(1,4,5)P_3$ in Ca^{2+} signalling is well established but the characteristics of $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores and other intracellular Ca^{2+} stores is, however, not entirely understood. The aim of the work presented in this thesis was to characterize intracellular Ca^{2+} stores and to investigate their properties in more detail.

In *chapter 2* it is shown that in addition to $Ins(1,4,5)P_3$, a GTP hydrolysing mechanism releases Ca^{2+} from intracellular Ca^{2+} stores. The action of GTP depends on the presence of polyethylene glycol and GTP acts on $Ins(1,4,5)P_3$-insensitive compartments. The polyethylene glycol-dependence of GTP-induced Ca^{2+} release has been shown for many cell types [Dawson and Comerford 1989; Gill et al. 1989]. In some cell types GTP does not induce Ca^{2+} release but potentiates the action of $Ins(1,4,5)P_3$. For the latter action it is suggested that GTP induces a coupling between intracellular Ca^{2+} stores by fusing membranes or by connecting Ca^{2+} stores. In this study no indications for a direct coupling between stores in the permeabilized acinar cell preparation were found. However, the observation that the size of the $Ins(1,4,5)P_3$-sensitive and the GTP-sensitive Ca^{2+} store are inversely related suggests that in intact cells these stores may be coupled to each other. The physiological relevance of this observation will be discussed below.

Chapter 3 deals with the effects of ruthenium red on intracellular Ca^{2+} stores. Ruthenium red is often used in studies with permeabilized cells to prevent Ca^{2+} uptake by mitochondria. This compound inhibits Ca^{2+}-ATPase activity when higher concentrations are used [Schuurmans Stekhoven and Bonting 1981]. In permeabilized pancreatic acinar cells a low concentrations of 5 μM ruthenium red completely prevents mitochondrial Ca^{2+} uptake activity without interfering Ca^{2+} uptake into non-mitochondrial pools. At concentrations exceeding 20 μM, however, ruthenium red inhibits Ca^{2+} uptake into non-mitochondrial Ca^{2+} stores. Interestingly, ruthenium red concentrations below 100 μM selectively prevent Ca^{2+} accumulation into $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores. At higher

concentrations the drug also inhibited Ca^{2+} uptake in GTP-sensitive stores. The action of ruthenium red was compared with that of vanadate and thapsigargin, two well known inhibitors of intracellular Ca^{2+}-ATPases. Ruthenium red induced a Ca^{2+} loss from steady-state loaded intracellular Ca^{2+} stores with a higher rate compared to the Ca^{2+} loss induced by intracellular Ca^{2+}-ATPase inhibitors. Therefore, it was concluded that the action of ruthenium red can not be explained by Ca^{2+}-ATPase inhibition alone. Thus far, the mechanism by which ruthenium red evokes the differential release of Ca^{2+} from the Ins(1,4,5)P_3-sensitive and the GTP-sensitive Ca^{2+} store is unclear, although it might be speculated that the drug acts with different potencies on the Ca^{2+} release channels of the respective stores. In conclusion, ruthenium red is a useful tool in permeabilized cells to deplete selectively Ins(1,4,5)P_3-sensitive stores.

In the study presented in *chapter 3*, thapsigargin was used to inhibit Ca^{2+}-ATPase activity on intracellular Ca^{2+} stores. Thapsigargin is a highly specific inhibitor of all known SERCA Ca^{2+}-ATPases [Lytton et al. 1991]. Due to its specificity and its membrane permeability, thapsigargin has become a popular tool to modulate Ca^{2+}-ATPase activity of intracellular Ca^{2+} stores in intact cells. In permeabilized acinar cells submaximal concentrations thapsigargin inhibit, in contrast to ruthenium red, the Ca^{2+} accumulation in all Ca^{2+} stores in an equal extent. Moreover, at a maximal inhibitory concentration, thapsigargin completely prevents Ca^{2+} uptake into intracellular Ca^{2+} stores. Therefore, all Ca^{2+} uptake in non-mitochondrial Ca^{2+} pools is provided by SERCA Ca^{2+}-ATPases. Subsequently, a heavy liver microsomal fraction was used to study the inhibitory action of thapsigargin on Ca^{2+}-stimulated ATP hydrolysis and ATP-dependent Ca^{2+} transport (*chapter 4*). Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activity (i.e. intracellular Ca^{2+}-ATPase activity) and Ca^{2+} transport activity were found to be inhibited by thapsigargin to the same extent. The Ca^{2+}-stimulated activities are, just as skeletal muscle Ca^{2+}-ATPase, inhibited in a non-competitive manner. Moreover, both the ATP hydrolysing and the transport activity have the same Ca^{2+} dependence and it can be concluded that both activities are strictly coupled and therefore represent the same enzyme activity.

It has been reported in literature that the high-affinity CCK receptor agonist JMV-180 can elicit repetitive increases in $[Ca^{2+}]_i$ without stimulating the hydrolysis of phosphatidylinositol 4,5-bisphosphate [Saluja et al. 1989; 1992; Yule and Williams 1992]. The importance of this finding is that it suggests the involvement of an intracellular messenger other than Ins(1,4,5)P_3 in the process of receptor-evoked Ca^{2+} release from intracellular stores. To investigate the existence of unknown second messengers in Ca^{2+} signalling, the effects of the putative phospholipase C inhibitor U73122 were studied in pancreatic acinar cells (*chapter 5*). During this study on both intact and permeabilized acinar cells, it became clear that this thiol reagent, in addition, depleted intracellular Ca^{2+}

stores. Interestingly, U73122 depleted, just like ruthenium red, selectively the Ins(1,4,5)P_3-sensitive Ca^{2+} store. It is not likely that U73122 inhibits Ca^{2+} pumps since the effects of thapsigargin and U73122 on charged Ca^{2+} stores were shown to be additive. Compared to ruthenium red, U73122 is a more interesting tool since the latter is membrane permeable. In intact cells U73122 displayed the same effects as in permeabilized cells since the drug depleted agonist-sensitive Ca^{2+} stores. Interestingly, in individual cells U73122 induced Ca^{2+} oscillations demonstrating that Ca^{2+} oscillations per se do not depend on the formation of Ins(1,4,5)P_3 but that depletion of the agonist-sensitive store together with influx of extracellular Ca^{2+} are a minimal requirement to induce repetitive and transient cytosolic Ca^{2+} rises. Thapsigargin was not able to induce these repetitive Ca^{2+} transients and the results with U73122 therefore indicate that the intactness of the Ins(1,4,5)P_3-insensitive compartments is essential for the generation of Ca^{2+} spikes.

In this thesis evidence is provided for the existence of a heterogeneous population of Ins(1,4,5)P_3-sensitive stores and that store heterogeneity determines the quantal features of Ins(1,4,5)P_3-induced Ca^{2+} release in acinar cells (*chapter 6*). Kinetic analysis of Ins(1,4,5)P_3-induced Ca^{2+} release in permeabilized pancreatic acinar cells revealed that stores, in contrast to hepatocytes [Oldershaw et al. 1991], are not depleted in an all-or-nothing fashion. After submaximal stimulation, a considerable amount of Ca^{2+} is retained within the store, due to compensatory Ca^{2+} uptake. It is proposed that store heterogeneity is the underlying principle of quantal Ca^{2+} release in permeabilized pancreatic acinar cells. A heterogeneous distribution of Ins(1,4,5)P_3-receptors and/or Ca^{2+} pumps is suggested to explain the observed results. Thus, stores containing a relative low number of Ins(1,4,5)P_3-receptors are only partially depleted during submaximal stimulation due to a compensatory pumping mechanism, whereas stores containing a relative high number of receptors are fully depleted under the same condition since the Ca^{2+} pumps can not compensate for the large Ca^{2+} efflux. The alternative explanation would be that different stores contain different receptor subtypes which display different sensitivities for Ins(1,4,5)P_3. In other studies it has been suggested that receptors with different sensitivities for Ins(1,4,5)P_3 explain the mechanism of Ins(1,4,5)P_3-induced Ca^{2+} release [Oldershaw et al. 1991]. In line with this suggestion, Parys et al. [1993] demonstrated the existence of several subtypes in smooth muscle. An argument against the involvement of different sensitivities between subtypes is that a narrow range of Ins(1,4,5)P_3 concentrations determines the dose-response curve for Ins(1,4,5)P_3-induced Ca^{2+} release. Moreover, Mauger and co-workers found in hepatocytes that only one binding site is involved in the regulation of Ins(1,4,5)P_3-induced Ca^{2+} release [Mauger et al. 1989; Pietry et al. 1990].

An alternative hypothesis to explain the 'quantal' type of Ins(1,4,5)P_3-stimulated

Ca^{2+} release, based on the intravesicular Ca^{2+} content regulating the sensitivity of the $Ins(1,4,5)P_3$ receptor to activation by $Ins(1,4,5)P_3$ rather than heterogeneity between $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores, has been proposed by Irvine **[1990]**. According to this model, the $Ins(1,4,5)P_3$ receptor sensitizes as the intravesicular Ca^{2+} content increases. In the study presented in *Chapter 6*, in which the intravesicular Ca^{2+} content was decreased by the action of either thapsigargin or ruthenium red, no evidence was found for such a regulatory mechanism to be operative in permeabilized pancreatic acinar cells.

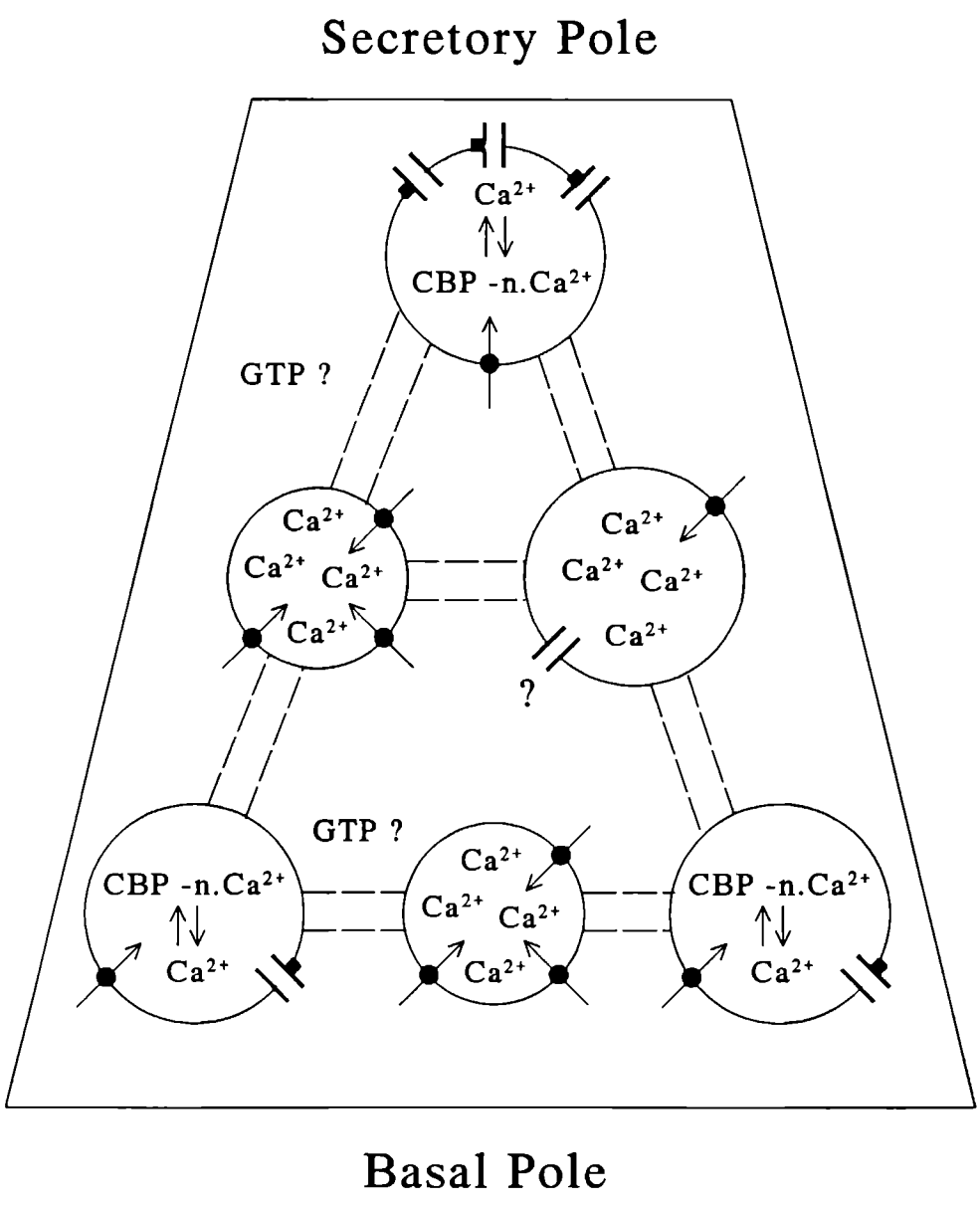
In a further study evidence is provided for a heterogeneous distribution of Ca^{2+} pump, storage and release sites in intracellular Ca^{2+} stores (*chapter 7*). Active Ca^{2+} uptake in the $Ins(1,4,5)P_3$-insensitive store could be demonstrated to be a first-order process reaching steady-state within 3 min. By contrast, active Ca^{2+} uptake in the $Ins(1,4,5)P_3$-sensitive store was found to be virtually linear with time during the first 4 min and did not reach steady-state before 7 min following its initiation by the addition of ATP. This deviation from a mono-exponential rate equation can be explained by the presence of a Ca^{2+} binding protein within this store buffering the intravesicular Ca^{2+} concentration. The presence of such a Ca^{2+} binding protein, referred to as calreticulin [Michalak et al. 1992], can also explain the observation that the Ca^{2+} uptake capacity of the $Ins(1,4,5)P_3$-sensitive store is higher than that of the $Ins(1,4,5)P_3$-insensitive store lacking this protein. The initial Ca^{2+} uptake rate was significantly higher in the $Ins(1,4,5)P_3$-insensitive store. This suggests that this store is either enriched in Ca^{2+} pumps or that it contains a distinct class of Ca^{2+} pumps characterised by a higher pumping rate.

Taken together, intracellular stores form a heterogeneous population of Ca^{2+} stores and the essential characteristics of the heterogenous store population are visualized in figure 1. In general three store types can be distinguished in this preparation: (i) the $Ins(1,4,5)P_3$-sensitive Ca^{2+} store (60 % of the total size) (ii) the GTP-sensitive Ca^{2+} store (20 %) and (iii) the $Ins(1,4,5)P_3$- and GTP-insensitive Ca^{2+} store (20 %). The possible function of the stores in Ca^{2+} oscillations and in Ca^{2+} wave propagation as well as the localization of the stores will be discussed below in more detail.

The two parallel lines with a closed box represent an Ins(1,4,5)P_3-operated Ca^{2+} channel. The two parallel lines with a question mark represent an unidentified Ca^{2+} channel; the possible candidates are the ryanodine receptor and the Ins(1,4,5)P_3 receptor. A closed circle with an arrow represent a Ca^{2+} pump. The Ins(1,4,5)P_3-sensitive stores contain Ca^{2+} storage proteins which can bind many Ca^{2+} ions. The presence of these proteins is abbreviated to CBP.

This model visualizes the suggested organization and interaction of intracellular Ca^{2+} stores in pancreatic acinar cells. The high Ins(1,4,5)P_3-sensitive stores responsible for the initial rise in the cytosolic Ca^{2+} concentration are located at the secretory pole of the cell and contain many receptors. The low Ins(1,4,5)P_3-sensitive stores contain only a few receptors and are located in the basolateral region of the cell. Both the high and the low sensitive stores are equiped with a Ca^{2+} storage system. The stores responsible for the propagation of the Ca^{2+} wave are found in between these two stores and contain the unidentified Ca^{2+} channel. Stores devoid of a release site are found throughout the cell and contain many Ca^{2+} pumps but are not equiped with a Ca^{2+} storage system. The dashed lines between the stores suggest a possible luminal continuity of all stores in intact acinar cells. The possible GTP-mediated interaction between Ins(1,4,5)P_3-insensitive and Ins(1,4,5)P_3-sensitive stores in intact cells is indicated in the figure. See text for more detail of the proposed model.

Figure 1 *Store heterogeneity in pancreatic acinar cells*



The function of the Ins(1,4,5)P_3-sensitive store in Ca^{2+} homeostasis is relatively clear, since initiation of Ca^{2+} oscillations is entirely due to the action of Ins(1,4,5)P_3. However, the mechanisms regulating Ins(1,4,5)P_3-induced Ca^{2+} release are still largely unknown. In this thesis it is shown that store heterogeneity is the underlying principle of quantal Ca^{2+} release. In light of the findings recently reported by Kasai et al. **[1993]** and Thorn et al. [1993] it is interesting to suggest that stores having a relatively high number of Ins(1,4,5)P_3-receptors are located at the apical pole whereas stores with a low receptor density are found in the basal pole of the cell. Thus, despite the fact that Ins(1,4,5)P_3 is generated at the basalateral face of the cell the Ca^{2+} rise can be initiated at the apical pole due to (i) the fast diffusion of the second messenger through the cytosol [Allbritton and Meyer 1993; Gromada et al. 1993] and (ii) the high Ins(1,4,5)P_3-sensitivity of these stores.

During global Ca^{2+} rises the signal originating in the apical pole is spreaded back towards the basal pole of the cell. The mechanism propagating the Ca^{2+} signal through the cell is suggested to be the Ca^{2+}-induced Ca^{2+} release mechanism. It is suggested that ryanodine receptors are responsible for this mechanism [Nathanson et al. 1992]. Although we have no indications for the existence of calcium-induced Ca^{2+} release (unpublished observations, see *chapter 3*), others provided evidence for such a mechanism in pancreatic acinar cells [Wakui et al. 1990; Dehlinger-Kremer et al. 1991; Nathanson et al. 1992, Kasai et al. 1993]. GTP- and Ins(1,4,5)P_3-insensitive Ca^{2+} stores may be a candidate responsible for wave propagation, provided that they contain ryanodine receptors. An alternative candidate for the propagating function may be Ins(1,4,5)P_3-sensitive Ca^{2+} stores themselves since cytosolic Ca^{2+} regulates Ins(1,4,5)P_3-receptor activity in a direct or indirect fashion [Pietry et al. 1990; Bezprozvanny et al. 1991; Finch et al. 1991; Zhang et al. 1993]. Ca^{2+} concentrations up to 600 nM potentiate (whereas higher concentrations inhibit) the mechanisms activated by Ins(1,4,5)P_3. Thus at low levels of Ins(1,4,5)P_3, Ins(1,4,5)P_3-induced Ca^{2+} release may be activated by Ca^{2+} when a Ca^{2+} wave arrives at the vicinity of the store. In conclusion, the receptor responsible for wave propagation is unknown, but it is very likely that the signal is carried by a Ca^{2+} dependent mechanism. Since releasable stores are localized throughout cytoplasm [Kasai et al. 1993] the Ca^{2+} wave is not a simple diffusion process. It is more likely that sequential release at discrete sites propagates the Ca^{2+} wave in manner comparable to the saltatory conduction of action potentials in vertebrate neurons. If involvement of the stores is ruled out by preventing Ca^{2+} release from these stores, a reduced wave speed is indeed observed [Nathanson et al. 1992].

At the basal pole stores are found with a low sensitivity for Ins(1,4,5)P_3 [Kasai et al. 1993]. In the model proposed in *chapter 6* low sensitive stores are suggested to contain a low density of Ins(1,4,5)P_3-receptors. The receptors of these stores are likely to

be opened since Ins(1,4,5)P_3 is generated at the basolateral side of the cell but remain largely filled due to a compensatory uptake mechanism. If the Ca^{2+} level in the environment of these stores is elevated, due to arrival of the Ca^{2+} wave, the open probability of the Ins(1,4,5)P_3-receptors will be increased (see above). As a consequence, these stores will be depleted since pumping can not compensate anymore for the increased permeability.

The nature of Ca^{2+} waves is transient and the mechanisms terminating Ca^{2+} release from stores is poorly understood. Release may be terminated as stores may come fully depleted or alternatively, the release process is inhibited by cytosolic Ca^{2+} reaching values at which the stimulatory effect of Ins(1,4,5)P_3 becomes abolished [Willems et al. 1990] by a mechanism that may involve the Ca^{2+}-dependent phosphorylation of the Ins(1,4,5)P_3 receptor [Zhang et al. 1993]. After termination of the release process, Ca^{2+} is extruded from the cytosol by plasma membrane Ca^{2+}-ATPases and intracellular Ca^{2+}-ATPases. Ca^{2+} waves are not only transient but are also repetitive and have the same amplitude. The Ins(1,4,5)P_3-insensitive stores contain a relative high pump capacity and likely play an important role in the quick removal of Ca^{2+} (*chapter 7*). By an unknown mechanism stores carrying the release sites become refilled and a new wave can be reinitiated since the Ins(1,4,5)P_3 receptors are resensitized by the low intracellular Ca^{2+} level. In *chapter 2* it is suggested that GTP controls the size of the Ins(1,4,5)P_3-sensitive store in intact cells. In *chapter 7* it is shown that Ins(1,4,5)P_3-insensitive stores contain the majority of the Ca^{2+} pumps. It is attemptive to re-evaluate the role of the GTP-sensitive store in Ca^{2+} signalling. This store may have (i) an important role in the removal of Ca^{2+} from the cytosol and (ii) a GTP hydrolysing mechanism which may regulate the flow of Ca^{2+} from the pumping sites to the release sites where an intravesicular storage system allows the accumulation of large amounts of Ca^{2+} at the vicinity of the Ins(1,4,5)P_3-operated Ca^{2+} channel.

The importance of Ins(1,4,5)P_3-insensitive stores and extracellular Ca^{2+} sources in Ca^{2+} oscillations is demonstrated in *chapter 5*. In intact cells U73122 induces oscillations by depleting selectively Ins(1,4,5)P_3-sensitive stores. The oscillations depend on intact Ins(1,4,5)P_3-insensitive stores since thapsigargin can only induce a single Ca^{2+} transient. Thus, after each rise Ca^{2+} is removed from the cytosol by Ins(1,4,5)P_3-insensitive stores and by plasma membrane Ca^{2+}-ATPases [Tepekin et al. 1992a, 1992b]. In addition, Ca^{2+} originating from extracellular sources has an important function in this process. During influx, Ca^{2+} is pumped into Ins(1,4,5)P_3-insensitive stores and is consequently released by an unknown mechanism. The latter mechanism may be Ca^{2+}-induced Ca^{2+} release.

The localization of the Ins(1,4,5)P_3-insensitive Ca^{2+} stores is unknown. If these stores have a function in the re-accumulation and redistribution of Ca^{2+} they have to be localized throughout the acinar cell. Apparently, these stores are localized in the

basolateral part of the cell since blockade of intracellular Ca^{2+} pumps results in rise of cytosolic Ca^{2+} in this region of the cell [Elliott et al. 1992]. In response to pump inhibition Ins(1,4,5)P_3-insensitive stores should be emptied faster than Ins(1,4,5)P_3-sensitive stores for two reasons. Ins(1,4,5)P_3-insensitive stores contain most of the Ca^{2+} pumps and more importantly these stores have a very high Ca^{2+} permeability (*chapter 7*).

Taken together, the exocrine acinar cell contains a heterogeneous population of intracellular Ca^{2+} stores. Whether these stores form a luminal continuity, as suggested for hepatocytes [Renard-Rooney et al. 1993], is unknown. Although these stores may form one compartment, stores may act as individual entities in intact cells due to the existence of Ca^{2+}, Ins(1,4,5)P_3 (and maybe additional) gradients. All stores apparently have a role in Ca^{2+} signalling but the interaction between the stores is still largely unknown. It is likely that the interaction between the stores and the regeneration of Ca^{2+} transients will be hot topics in future research.

Chapter 9

Samenvatting

De alvleesklier is een orgaan met een exocriene en een endocriene functie. Het grootste deel van de pancreas bestaat uit exocrien weefsel. De exocriene cellen zijn verantwoordelijk voor de aanmaak en uitscheiding van een vloeistof rijk aan spijsverteringsenzymen en bicarbonaat. De endocriene cellen zijn gegroepeerd in de eilandjes van Langerhans. Deze eilandjes liggen ingebed in het exocriene weefsel en worden door het hele orgaan heen aangetroffen. De endocriene cellen zijn betrokken bij de regulatie van de suikerspiegel in het lichaam [Bockman 1993, Kern 1993, Korc 1993].

De acineuze cellen van de exocriene pancreas produceren spijsverteringsenzymen en verzorgen een gedeelte van de vloeistofsecretie. De acineuze cellen liggen gegroepeerd in een "braamvormige" structuur, welke acinus genoemd wordt, en omsluiten een holte die acineuze holte genoemd wordt. (Acinus is het latijnse woord voor braam). De aangemaakte produkten worden uitgestort in deze holte en worden van daaruit via een gangenstelsel afgevoerd naar de twaalfvingerige darm. De wanden van dit wijdvertakte gangenstelsel worden gevormd door de ductulaire cellen welke het hoofdbestanddeel van de bicarbonaatrijke vloeistofuitscheiding verzorgen. De uitgescheiden vloeistof zorgt er onder andere voor dat de spijsverteringsenzymen, afkomstig uit de vele acini, afgevoerd worden naar de darmen en dat het maagzuur geneutraliseerd wordt [Bockman 1993, Case and Argent 1993, Kern 1993, Petersen 1993].

Het peptidehormoon cholecystokinine (CCK) en de neurotransmitter acetylcholine (ACh) zijn de belangrijkste stimulatoren van het proces van enzymsecretie. Beide hydrofiele messengers binden aan specifieke receptoren op de plasmamembraan. Activering van deze receptoren leidt tot de vorming van intracellulaire boodschappers. Met andere woorden, de informatie van de agonist wordt vertaald in een voor de cel begrijpelijk signaal. ACh en CCK receptoren werken beiden via de fosfolipase-C route. Receptoractivering leidt door tussenkomst van een G-eiwit tot een verhoging van de activiteit van het fosfolipase C. Dit effectorenzym splitst het plasmamembraanfosfolipide fosfatidylinositol-bisfosfaat in een tweetal second messengers. De gevormde boodschappers zijn het lipofiele diacylglycerol en het hydrofiele inositol (1,4,5)-trisfosfaat ($Ins(1,4,5)P_3$). Diacylglycerol blijft in de plasmamembraan en activeert daar proteïne kinase C. $Ins(1,4,5)P_3$ diffundeert in het cytoplasma en maakt Ca^{2+} vrij uit in de cel gelegen opslagplaatsen. Beide wegen leiden tot de fosforylering van vele eiwitten. De rol van de gefosforyleerde produkten is onduidelijk maar het is waarschijnlijk dat ze belangrijk zijn bij de regulatie van enzymsecretie. Uit veel onderzoek is in ieder geval duidelijk geworden dat zowel de activering van proteine kinase C als de verhoging van de cytosolaire Ca^{2+} concentratie noodzakelijk zijn om het proces van enzymsecretie te stimuleren. De exacte mechanismen die leiden tot enzymsecretie zijn echter nog steeds niet opgehelderd [Gardner and Jensen 1993; Petersen 1993; Williams and Yule

1993].

Gedurende maximale stimulering van acineuze cellen door ACh of CCK treedt een snelle maar tijdelijke stijging op van de gemiddelde cytosolaire Ca^{2+} concentratie van 0.1 μM naar 0.5 tot 1.0 μM. Na deze transiente verhoging daalt de Ca^{2+} spiegel tot een waarde die iets hoger is dan de rustwaarde. Het Ca^{2+} dat verantwoordelijk is voor de transiente Ca^{2+} stijging is afkomstig uit een in de cel gelegen Ca^{2+} opslagplaats. Het continue verhoogde niveau na de eerste korte stijging wordt alleen waargenomen indien in het extracellulair medium Ca^{2+} aanwezig is. Wanneer cellen submaximaal gestimuleerd worden, wordt het Ca^{2+} niveau in de cellen periodisch verhoogd. Ook bij deze Ca^{2+} "oscillaties" spelen intracellulaire Ca^{2+} opslagplaatsen een zeer voorname rol. Hoe de oscillaties gegenereerd worden is slechts gedeeltelijk opgehelderd. Het is in ieder geval duidelijk dat Ins(1,4,5)P_3-gevoelige opslagplaatsen verantwoordelijk zijn voor de initiatie van deze oscillaties [Petersen 1993, Williams and Yule 1993].

Uit recent onderzoek is gebleken dat een Ca^{2+} stijging zich als een golf door de cel verplaatst [Kasai and Augustine 1990]. De eerste Ca^{2+} stijging vindt plaats aan de apicale zijde van de cel waarna het signaal zich verspreidt in de basolaterale richting. De initiële stijging in het apicale gebied van de cel wordt door Ins(1,4,5)P_3 veroorzaakt. Het signaal verspreidt zich doordat Ca^{2+} vrijgemaakt wordt uit opslagplaatsen volgens het "Ca^{2+}-induced Ca^{2+} release" mechanisme. Volgens dit mechanisme worden Ca^{2+} kanalen op de opslagplaatsen geactiveerd door een verhoogde cytosolaire Ca^{2+} concentratie. Het is onduidelijk of hierbij de Ins(1,4,5)P_3 receptoren zelf of de verwante Ins(1,4,5)P_3-ongevoelige ryanodine receptoren betrokken zijn, omdat beide kanalen gevoelig zijn voor cytosolair Ca^{2+}. In het basolaterale gebied bevinden zich, net als in het apicale gebied, Ins(1,4,5)P_3-gevoelige opslagplaatsen. De opslagplaatsen in het basolaterale gebied zijn blijkbaar ongevoeliger voor Ins(1,4,5)P_3 omdat de Ca^{2+} stijging niet in dit gebied geinitiëerd wordt [Nathanson et al. 1992; Gromada et al. 1993; Kasai et al. 1993; Thorn et al. 1993]. De Ins(1,4,5)P_3-gevoelige opslagplaatsen in het basolaterale gebied van de cel moeten inderdaad ongevoeliger zijn omdat hier receptor-gestimuleerde Ins(1,4,5)P_3 produktie plaatsvindt [Rozenzweig et al. 1983]. Dat cytosolair Ca^{2+} toch als eerste in het apicale gebied kan stijgen wordt mogelijk gemaakt doordat Ins(1,4,5)P_3 zich zeer snel door het cytosol kan verplaatsten [Allbritton and Meyer 1993; Gromada et al. 1993]. De hierboven besproken studies maar ook morfologische en celfractioneringsstudies laten zien dat een heterogene populatie van opslagplaatsen aanwezig is in de acineuze cel [Volpe et al. 1988, Hashimoto et al. 1988, Dehlinger-Kremer et al. 1989, Sharp et al 1992].

Het belang van intracellulaire Ca^{2+} opslagplaatsen in het proces van signaaloverdracht is hierboven in het kort uiteengezet. Een nadeel van het gebruik van intacte cellen om deze opslagplaatsen te bestuderen is de barrière die door de plasmamembraan gevormd wordt. Om een betere toegang tot intracellulaire opslagplaatsen te verkrijgen wordt vaak gebruik gemaakt

van het gepermeabiliseerde celsysteem. Met deze methode worden gaten in de plasmamembraan gemaakt zonder dat daarbij intracellulaire structuren worden aangetast. Hierdoor wordt een goede controle verkregen over de cytosolaire omgeving van de opslagplaatsen. Tevens kunnen gemakkelijk stoffen als Ins(1,4,5)P_3 toegediend worden die normaal de plasmamembraan niet passeren. Met deze methode werd voor het eerst aangetoond dat Ins(1,4,5)P_3 Ca^{2+} vrijmaakt uit niet-mitochondriële intracellulaire Ca^{2+} opslagplaatsen [Streb et al. 1983]. Het belang van Ins(1,4,5)P_3 in signaaloverdracht is sindsdien vastgesteld in vele celtypen. De eigenschappen van intracellulaire Ca^{2+} opslagplaatsen en het mechanisme van Ins(1,4,5)P_3-geïnduceerde Ca^{2+} release zijn slechts gedeeltelijk opgehelderd. In dit proefschrift is het lekke celsysteem gebruikt om deze vraagstellingen te onderzoeken.

In hoofdstuk 2 worden de effecten van GTP op intracellulaire Ca^{2+} opslagplaatsen beschreven. Tijdens deze studie bleek dat naast Ins(1,4,5)P_3, ook GTP Ca^{2+} kan vrijmaken uit niet-mitochondriële Ca^{2+} opslagplaatsen. Gevonden werd dat de hydrolyse van GTP daarbij noodzakelijk is en het effect bleek alleen bereikt te kunnen worden in aanwezigheid van polyethyleenglycol. Bij andere celtypen (maar niet alle) is gebleken dat GTP zelf geen release induceert maar de effecten van Ins(1,4,5)P_3 versterkt [Dawson and Comerford 1989, Gill et al. 1989]. In de exocriene pancreas kon hiervoor geen direct bewijs worden gevonden omdat de effecten van GTP en Ins(1,4,5)P_3 onafhankelijk van elkaar bleken te zijn. Desondanks werden met het lekke celsysteem aanwijzingen gevonden voor het bestaan van een mogelijke koppeling van GTP-gevoelige en Ins(1,4,5)P_3-gevoelige opslagplaatsen in intacte cellen. De grootte van de GTP-gevoelige opslagplaatsen bleek namelijk omgekeerd evenredig te zijn met die van de Ins(1,4,5)P_3-gevoelige opslagplaats.

De effecten van ruthenium rood worden beschreven in hoofdstuk 3. Normaal worden lage concentraties van deze kleurstof gebruikt om mitochondriële Ca^{2+} opname te voorkomen. De reden om mitochondriële opnameremmers te gebruiken is dat de Ins(1,4,5)P_3-gevoelige opslagplaatsen niet in deze organellen gelegen zijn. Bij hogere concentraties werd gevonden dat ruthenium rood echter selectief de stapeling van Ca^{2+} in Ins(1,4,5)P_3-gevoelige opslagplaatsen verhinderde zonder die in andere niet-mitochondriële opslagplaatsen aanmerkelijk te beïnvloeden. In de literatuur is het beschreven dat hogere concentraties ruthenium rood Ca^{2+} pompen kunnen remmen [Schuurmans Stekhoven and Bonting 1981]. Daarom werd de remming door ruthenium rood vergeleken met die van bekende remmers van Ca^{2+} pompen. Uit deze vergelijking werd geconcludeerd dat remming van intracellulaire Ca^{2+} pompen niet het enige mechanisme kan zijn waardoor ruthenium rood zijn effect bereikt.

Thapsigargine was een van de Ca^{2+} pomp remmers die werd gebruikt in hoofdstuk 3. Thapsigargine is een zeer specifieke remmer van alle bekende intracellulaire Ca^{2+}-ATPases van de SERCA Ca^{2+}-ATPase familie [Lytton et al. 1991]. In tegenstelling tot

ruthenium rood, bleek thapsigargin bij sub-optimale concentraties de opname van Ca^{2+} in alle opslagplaatsen in gelijke mate te remmen. Volledige remming van de opname werd verkregen bij de maximaal effectieve concentratie van thapsigargine, hetgeen betekent dat alle Ca^{2+} opname in intracellulaire opslagplaatsen door SERCA Ca^{2+}-ATPases verzorgt wordt. Het remmingsmechanisme van thapsigargine werd in meer detail onderzocht door gebruik te maken van een microsomale fractie geïsoleerd uit de rattelever (hoofdstuk 4). De remmende werking van thapsigargine werd onderzocht op zowel de enzymatische activiteit (Ca^{2+}-gestimuleerde ATP hydrolyse) als de transportactiviteit van de intracellulaire Ca^{2+} pompen. Omdat Ca^{2+} pompen door Ca^{2+} gestimuleerd worden in het submicromolaire concentratie gebied werd de remming onderzocht bij diverse Ca^{2+} concentraties in dit gebied. In hoofdstuk 4 wordt aangetoond dat thapsigargine (i) beide activiteiten van de Ca^{2+} pomp in gelijke mate remt en (ii) dat de remming dosisafhankelijk is en (iii) dat de remming non-competitief is (d.w.z niet wordt opgeheven bij hogere Ca^{2+} concentraties).

In hoofdstuk 5 worden de effecten beschreven van de recent ontwikkelde fosfolipase C remmer, U73122. Deze stof werd zowel in intacte als in lekke cellen bestudeerd. In beide celpreparaten bleek U73122 de Ins(1,4,5)P_3-gevoelige opslagplaatsen te depleteren. De werking van deze stof is dus vergelijkbaar met die van ruthenium rood. U73122 biedt echter de mogelijkheid dat het aangewend kan worden in intacte cellen. Een interessante bevinding in deze studie is dat U73122 Ca^{2+} oscillaties bleek te kunnen inducereren in acineuze cellen. De vorming van Ins(1,4,5)P_3 lijkt daarmee niet per sé nodig te zijn om het cytosolaire Ca^{2+} niveau te laten oscilleren. In tegenstelling tot U73122 kon thapsigargine slechts één transiente Ca^{2+} stijging veroorzaken. Men kan daaruit concluderen dat intacte Ins(1,4,5)P_3-ongevoelige opslagplaatsen een minimale voorwaarde zijn voor het optreden van U73122-geinduceerde Ca^{2+} oscillaties in acineuze pancreascellen.

In hoofdstuk 6 wordt het onderzoek beschreven naar de mechanismen van Ins(1,4,5)P_3-geïnduceerde Ca^{2+} afgifte. Eerder werd reeds vermeld dat het mechanisme van Ins(1,4,5)P_3-geïnduceerde Ca^{2+} afgifte slechts gedeeltelijk begrepen wordt. Een interessante eigenschap van deze afgifte is dat suboptimale concentraties Ins(1,4,5)P_3 discrete hoeveelheden Ca^{2+} vrijmaken uit intracellulaire opslagplaatsen [Muallem et al. 1989]. Het is gebleken dat ondanks het feit dat er geen receptordesensibilisering optreedt, de opslagplaatsen niet volledig gedepleteerd worden [Meyer and Stryer 1990]. In hepatocyten wordt verondersteld dat verschillende opslagplaatsen receptoren bezitten met verschillende gevoeligheden voor Ins(1,4,5)P_3. Hierdoor worden bij suboptimale concentraties Ins(1,4,5)P_3 de meest gevoelige opslagplaatsen compleet geleegd en blijven de minder gevoelige opslagplaatsen onaangetast [Oldershaw et al. 1991]. In tegenstelling tot de hepatocyt, blijft er na sub-optimale stimulatie nog een aanzienlijke hoeveelheid Ca^{2+} achter in de Ins(1,4,5)P_3-gevoelige opslagplaatsen in gepermeabiliseerd acineuze cellen. In de acineuze cel wordt daarom gesteld dat de verhouding tussen de aantallen Ins(1,4,5)P_3 receptoren en Ca^{2+}

pompen op een opslagplaats de afgiftekarakteristiek van deze opslagplaats bepaalt. Dus opslagplaatsen met relatief veel receptoren zullen leeg zijn na sub-optimale stimulering. Opslagplaatsen met relatief weinig receptoren blijven gedeeltelijk gevuld omdat de pompen compenseren voor de Ca^{2+} uitstroom door de Ins(1,4,5)P_3-gecontroleerde Ca^{2+} kanalen. Uit deze studie wordt geconcludeerd dat Ins(1,4,5)P_3-gevoelige opslagplaatsen een heterogene populatie zijn.

Tenslotte wordt in hoofdstuk 7 aangetoond dat ook andere eigenschappen van Ca^{2+} opslagplaatsen niet homogeen verdeeld zijn over de opslagplaatsen. Het blijkt dat de meeste Ca^{2+} pompen zich bevinden in Ins(1,4,5)P_3-ongevoelige compartimenten. Ondanks het feit dat de Ins(1,4,5)P_3-ongevoelige opslagplaatsen de meeste pompcapaciteit bezitten, zijn het juist de Ins(1,4,5)P_3-gevoelige opslagplaatsen die de grootste hoeveelheid Ca^{2+} opstapelen. Het is daarom aannemelijk dat Ins(1,4,5)P_3-gevoelige opslagplaatsen uitgerust zijn met een Ca^{2+} bufferend systeem. Vele morfologische studies als ook vele celfractioneringsstudies wekken de indruk dat Ins(1,4,5)P_3-gevoelige opslagplaatsen uitgerust zijn met Ca^{2+} bindende eiwitten [Michalak et al. 1992]. Het recent gekarakteriseerde eiwit calreticulin is een serieuze kandidaat voor deze functie omdat dit eiwit in staat is grote hoeveelheden Ca^{2+} te binden met een lage affiniteit.

Samengevat kan geconcludeerd worden dat intracellulaire Ca^{2+} opslagplaatsen een heterogene populatie van opslagplaatsen zijn. Allereerst kunnen Ins(1,4,5)P_3-gevoelige en Ins(1,4,5)P_3-ongevoelige opslagplaatsen onderscheiden worden. De Ins(1,4,5)P_3-ongevoelige opslagplaatsen kunnen weer onderverdeeld worden in GTP-gevoelige en GTP-ongevoelige opslagplaatsen. Of een gedeelte van de opslagplaatsen door Ca^{2+}-induced Ca^{2+} release geleegd kan worden is onduidelijk. Er zijn pogingen ondernomen in hoofdstuk 3 om aanwijzingen te vinden voor de aanwezigheid ryanodine receptoren, maar het resultaat was negatief.

Verder kan gesteld worden dat de verschillende opslagplaatsen op verschillende wijzen beïnvloed kunnen worden door een aantal stoffen. Zowel ruthenium rood (hoofdstuk 3) als U73122 (hoofdstuk 5) kunnen selectief de opname van Ca^{2+} in Ins(1,4,5)P_3-gevoelige opslagplaatsen verhinderen. Vooral U73122 blijkt nuttig te zijn omdat deze stof membraandoorlaatbaar is en dus ook bij intacte cellen toegepast kan worden.

Uit hoofdstuk 6 en 7 blijkt dat niet alleen op basis van gevoeligheden voor second messengers of andere stoffen een onderscheid gemaakt kan worden tussen opslagplaatsen. Receptordichtheden (hoofdstuk 6), pompdichtheden (hoofdstuk 6 en 7) en opslagcapaciteiten (hoofdstuk 7) blijken niet gelijk verdeeld te zijn over de diverse opslagplaatsen. Uit de analyse van het verloop van Ins(1,4,5)P_3-geïnduceerde Ca^{2+} release is geconcludeerd dat de verhouding tussen de aantallen Ins(1,4,5)P_3-receptoren en Ca^{2+} pompen de gevoeligheid van een opslagplaats voor Ins(1,4,5)P_3 bepaald. De recente bevindingen van Kasai en medewerkers [1993] en van Thorn en medewerkers [1993] laten zien dat de gevoeligste

opslagplaatsen voor Ins(1,4,5)P_3 zich aan de apicale zijde van de cel bevinden en dat de minder gevoelige opslagplaatsen zich aan de basolaterale zijde van de cel bevinden. Het is daarom interessant te postuleren dat opslagplaatsen met relatief veel Ins(1,4,5)P_3 receptoren zich aan de apicale kant van de cel bevinden, terwijl opslagplaatsen met relatief weinig Ins(1,4,5)P_3-receptoren zich in het basolaterale gebied van de cel bevinden. Uit hoofdstuk 7 blijkt dat Ins(1,4,5)P_3-ongevoelige opslagplaatsen een geringe opnamecapaciteit hebben en veel Ca^{2+} pompen bezitten. Daarentegen bezitten Ins(1,4,5)P_3-gevoelige opslagplaatsen een grote opslagcapaciteit ondanks het feit dat ze met relatief weinig pompen uitgerust zijn. De opslagcapaciteit kan door intravesiculaire Ca^{2+}- bindende eiwitten verzorgd worden. Deze eigenschap maakt het mogelijk een grote hoeveelheid Ca^{2+} te concentreren bij plaatsen waar release plaatsvindt. Zoals boven al vermeld werd is het onduidelijk of in intacte cellen GTP-gevoelige opslagplaatsen in verbinding staan met Ins(1,4,5)P_3-gevoelige opslagplaatsen. In hepatocyten zijn recent aanwijzingen gevonden dat alle opslagplaatsen inderdaad met elkaar in contact staan [Renard-Rooney et al. 1993]. In dit geval kunnen in acineuze cellen de ongevoelige opslagplaatsen na een Ca^{2+} stijging in het cytosol, zorgen voor een snelle verwijdering van Ca^{2+}. Na deze snelle verwijdering wordt het gestapelde Ca^{2+} doorgesluisd naar de Ins(1,4,5)P_3-gevoelige gedeelten alwaar het door de Ca^{2+}-bindende eiwitten opgeslagen wordt. Door een artefact dat optreedt tijdens het lek maken van levercellen ontstaan er vele individuele compartimenten [Renard-Rooney et al. 1993]. Of dit het geval is in de acineuze cel is onduidelijk. Kortom, wat de exacte betekenis is van de intracellulaire opslagplaatsen in signaaloverdracht en hoe ze onderling in verhouding staan met elkaar is nog steeds niet opgehelderd. Het moge duidelijk zijn dat vraagstellingen rondom de betekenis van intracellulaire Ca^{2+} opslagplaatsen een belangrijk aandachtspunt zullen blijven bij toekomstig onderzoek naar signaaltransductiemechanismen.

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Dankwoord

Het onderzoek dat in deze dissertatie beschreven is, is uiteraard niet alleen door mij uitgevoerd.

Allereerst wil ik Joost Hoenderop bedanken voor zijn grote inzet en goede praktische vaardigheden. Verder wil ik Lizanne Donkers, Lex Theuvenet en wijlen Gea Visser bedanken voor de prettige samenwerking. Ook wil ik de bijdragen Remco Bosch, Richard Engbersen, Ilonka van Hoof en Tino Vermegen niet onvermoemd laten.

Further I would like to thank Peter Nàgy for his valuable contribution despite his short stay of two months in Nijmegen.

Voor de goede zorgen voor de proefdieren ben ik Albert Peters en Martin Faassen veel dank verschuldigd. Zeker mag ik niet vergeten Peter Willems te bedanken voor de vele middagen die hij met mij rond de filtreertrommel doorgebracht heeft. Daarnaast wil ik hem ook nog bedanken voor de vele en uitvoerige discussies en voor zijn belangstelling op het persoonlijke vlak.

De stichting 15th IUB Congress wil ik bedanken voor de beurstoekenning die het mij mogelijk maakte om aan het 21e FEBS Congress (1992) in Dublin deel te nemen.

I gratefully acknowledge the European Pancreatic Club for providing me a young investigator travel scholarship which allowed me to join the Paris meeting in 1993.

Mijn ouders ben ik zeer erkentelijk voor hun ondersteuning en stimulering tijdens mijn studie en mijn promotieonderzoek.

Als laatste wil ik Christa bedanken voor haar ondersteuning en voor het geduld dat ze met name tijdens de afronding van mijn proefschrift heeft moeten opbrengen. Maar vooral wil ik haar bedanken voor de twee schatten van kinderen die ze mij geschonken heeft.

Louiset, E., Van de Put, F.H.M.M., Tonon, M.C., Basille, C., Jenks, B.G., Vaudry, H. and Cazin, L. (1990) Electrophysiological evidence for the existence of $GABA_A$ receptors in cultured frog melanotrophs. Brain Research, **517**, 151-156.

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Curriculum Vitae

Frans van de Put werd geboren op 5 augustus 1965 te Oss. Na zes schooljaren op het Titus Brandsma Lyceum te Oss werd het VWO diploma in 1983 behaald. Na één studiejaar Scheikunde in 1984 werd aangevangen met de studie Biologie aan de Universiteit van Nijmegen. In 1985 behaalde de schrijver de propedeuse en in november 1989 studeerde hij af. Het hoofdvak werd doorlopen in Nijmegen op de afdeling Experimentele Dierkunde bij Dr. B.G. Jenks en de bijvakstage werd verricht gedurende 10 maanden aan de "Université de Rouen" in Frankrijk bij Dr. L. Cazin van de "Groupe de Recherche en Endocrinologie Moléculaire".

Van 1 december 1989 tot en met 30 november 1993 was hij werkzaam op de afdeling Biochemie van de Medische Faculteit van de Katholieke Universiteit Nijmegen. Gedurende deze periode werd het promotieonderzoek uitgevoerd onder leiding van Prof. Dr. J.J.H.H.M. de Pont en Dr. P.H.G.M. Willems.

Per april 1994 is hij begonnen als post-doc medewerker "in the Department of Physiology of the University of Manchester" in Engeland bij Dr. A.C. Elliott en Prof. Dr. R.M. Case.

Stellingen behorende bij het proefschrift

Characterization of Intracellular Calcium Stores in the Exocrine Pancreas

F.H.M.M. van de Put

I

De door Thorn et al. gebruikte concentratie ruthenium rood is veel te hoog om alleen "calcium-induced calcium release" te verhinderen.

Thorn, P., Gerasimenko, O. and Petersen O.H. (1994) EMBO Journal, 13, pp. 2038 - 2043.

Van de Put, F.H.M M., Hoenderop, J.H.J., De Pont, J.J.H.H.M. and Willems, P.H.G.M. (1993) J Membr. Biol , 135, pp. 153-163.

II

Het is nog steeds niet duidelijk of caffeine-induced calcium release, cyclic-ADP ribose-induced calcium release en calcium-induced calcium release allen gemedieerd worden door de ryanodine receptor in acineuze cellen

III

Intacte inositol-trisfosfaat ongevoelige calciumopslagplaatsen zijn een minimale voorwaarde voor het optreden van calciumoscillaties in de exocriene pancreascel

Willems, P.H.G M., Van de Put, F.H.M.M., Engbersen, R., Bosch, R R., Van Hoof, H.J.M , De Pont, J.J.H.H M (1994) Eur. J. Physiol., 427, pp. 233-243.

IV

Het door Marshall en Taylor vastgestelde effect van de intravesiculaire calciumconcentratie op de het mechanisme van inositol-trisfosfaat geinduceerde calcium release in hepatocyten is slechts schijnbaar aanwezig.

Marshall, I C B. and Taylor, C.W. (1993) Journal of Biological Chemistry, 268, 13214 -13220.

V

Remming van de calciumpomp resulteert in een zeer vertraagde efflux van calcium uit calcium-pyrophosphaat geladen intracellulaire opslagplaatsen in gepermeabiliseerde hepatocyten. Vanwege deze trage dissociatie mogen Oldershaw et al. niet zonder meer concluderen worden dat er geen (gedeeltelijke) desensibilisering van de inositol-trisfosfaat receptor optreedt gedurende aanhoudende stimulering van deze receptor.

Oldershaw, K.A., Richardson, A and Taylor, C.W (1992) Journal of Biological Chemistry, 267, 16312 - 16316.

VI

De regulatie van de inositol-trisfosfaat receptor door cytosolair calcium is een complex proces.

VII

De lage vergroting gebruikt door Nathanson et al. bij de localisatiestudie naar de inositol-trisfosfaat receptor in de acineuze pancreascel zal vermoedelijk niet te wijten zijn aan het zoek geraakt zijn van het objectief met een grotere vergroting gebruikt in de vorige studie.

Nathanson et al. (1994) Journal of Biological Chemistry, 269, pp 4693 - 4696.

Nathonson et al. (1992) Journal of Biological Chemistry, 267, pp. 18118 -18121.

VIII

De ijking van "calciumprogramma's" aan biologische parameters is zeker zo belangrijk als de ijking van zulke programma's aan fysische parameters

IX

Het verdient de aanbeveling het caffeinegebruik in een wetenschappelijke omgeving te beperken tot de dagelijkse dosis koffie.

X

De volledige naam voor een PC, "Personal Computer" moet niet al te persoonlijk opgevat worden.

XI

Indien pubs in Dublin aangemerkt mogen worden als een toeristische attractie, kan gesteld worden dat Dublin een aantrekkelijke vakantiebestemming is.

XII

De huidige tarieven van de British Rail mogen gezien worden als een nieuwe variant van "the Great Train Robbery".

XIII

Dat continentale wasmachines zonder aanpassingen aangesloten kunnen worden aan Britse waterleidingen is een grote stap voorwaarts in het Europese eenwordingsproces